ASSAY DEVELOPMENT, TISSUE DISTRIBUTION AND PHARMACODYNAMICS OF A NOVEL ESTROGEN-CHEMICAL DELIVERY SYSTEM FOR THE BRAIN

BY

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To my parents for their encouragement and unwavering
support toward my education, and my wife, Mahbobeh, and my son, Ehsan,
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Enhanced delivery and sustained release of estradiol (E_2) in the brain is desirable for effective treatments of the menopausal hot flush, prostatic adenocarcinoma, and fertility regulation. Our studies thus evaluated an E_2-chemical delivery system (E_2-CDS) for the brain, which is based upon the interconvertible dihydropyridine $\Leftrightarrow$ pyridinium ion redox reaction. The E_2-CDS requires multiple, facile chemical conversions, including the oxidation of E_2-CDS to the corresponding quaternary ion (E_2-Q^+), which provides the basis of locking the molecule within the brain, and the subsequent slow hydrolysis of E_2-Q^+ by esterases to E_2 in that tissue.

Initially, studies were undertaken to develop a reliable, specific, and sensitive method to simultaneously measure E_2-Q^+ and E_2 (two metabolites

of E_2-CDS) in various biological tissues. This method utilized the following steps: (1) selective solvent extraction of E_2-Q^+ and E_2 from the tissues; (2) base-catalyzed hydrolysis of E_2-Q^+ to E_2 in NaOH; (3) solid-phase purification of E_2 with C_{18} reversed-phase extraction columns; and (4) radioimmunoassay of E_2.

Subsequently, the *in vivo* tissue distributions of E_2-Q^+ and E_2 were determined in both male and female rats. The results revealed that the disappearance of E_2-Q^+ as well as E_2 was slow in brain tissue with a $t_{1/2}$ = 8-9 days. By contrast, both of these metabolites exhibited relatively rapid clearance from the plasma, liver, lung, kidney, heart, fat, and uterus.

After documenting the kinetic behaviors of E_2-CDS, time-course studies were then conducted to assess the dynamic effects of E_2-CDS on responses which are known to be affected by E_2. The E_2-CDS consistently exhibited prolonged and sustained suppression of pituitary gonadotropins secretion, i.e. LH and FSH in a dose- and time-dependent manner.

Finally, the therapeutic potentials of E_2-CDS were investigated in male and female rats. Studies in the male rat demonstrated that E_2-CDS is as effective as castration in both suppressing the plasma testosterone levels and reducing the weights of androgen-responsive tissues. Further studies in the female rat, examining the effects of E_2-CDS on tail-skin temperature (TST) responses, revealed that E_2-CDS can significantly attenuate the rise in TST.

Collectively, the results of these studies are consistent with the proposed mechanism of this drug delivery system, that is, the preferential retention of E_2-Q^+ by the brain, and the subsequent slow release of E_2 locally in that tissue. Furthermore, the profound pharmacodynamic effects of this delivery system support the view that E_2-CDS may be potentially useful for fertility regulation, the effective treatments of prostatic cancer, and certain brain-mediated estrogen withdrawal symptoms, i.e. menopausal hot flushes.

CHAPTER 1
INTRODUCTION

Estrogens exhibit a myriad of important regulatory roles in the growth, development, and maintenance of the structures and functions which are necessary for the continuation of the species. Their therapeutic applications for certain clinical problems have been appreciated since the turn of the century, when ovarian grafts were shown to prevent uterine atrophy and loss of sexual function in castrated animals (Knauer, 1900). Estrogen hormones have broad therapeutic applications and in most cases the steroids are used primarily for their central actions (Meites & Nicoll, 1965). Among these are the reproductive-related applications, including fertility regulation, sexual dysfunction, and the replacement therapy in postmenopausal patients; and the non-reproductive applications, including treatment of postmenopausal depression and cancer therapy. Nevertheless, the full spectrum of potential clinical benefits and applications of estrogen therapy has yet to be uncovered.

Physiologically, estrogen hormones exert two modes of action on the central nervous system (CNS), particularly on the brain. First, during the critical period of fetal/neonatal life, estrogens affect permanently some features of the brain structure and function which result in neuronal differentiation (Allen et al., 1989; Goy & McEwen, 1980). Second, during the course of adult life, these hormones exert their effects in a modulatory and reversible mode that influence a myriad of adult brain functions (McEwen, 1988; McEwen & Parsons, 1982).

Therapeutically, this latter central action of estrogens is of significant interest due to the existence of several clinical conditions which are influenced only by the presence of estrogens in the brain. For instance, after menopause the decline in ovarian function leads to a number of central nervous system (CNS)-mediated estrogen-withdrawal symptoms (Notelovitz, 1986). The symptoms are clearly caused by brain deprivation of estrogen (Judd, 1983; Lauritzen, 1973, 1982) since they can be alleviated by the replacement of estrogen (Campbell & Whitehead, 1977; Upton, 1984). Furthermore, evidence suggests that the brain is the primary locus where estradiol (E_2) exerts its effect to inhibit the secretion of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus (Goodman & Knobil, 1981; Kalra & Kalra, 1980, 1983, 1989; Plant, 1986) and hence of LH from the anterior pituitary and eventually of gonadal steroid hormones. As such, the E_2 hormone has been and continues to be used therapeutically for (1) fertility regulation (Briggs, 1976; Davidson, 1969) and (2) treatment of androgen-dependent prostatic adenocarcinoma (van Steenbrugge et al., 1988) by virtue of suppressing plasma androgen levels (Carlstrom et al., 1989). Additionally, estrogens are believed to act centrally to stimulate male and female sexual behaviors (Beyer et al., 1976; Christensen & Clemens, 1974; MacLusky et al., 1984), to regulate body weight (Palmer & Gray, 1986; Pliner & Fleming, 1983), and may have influences on mood (Klaiber et al., 1976, 1979; Lauritzen & van Keep, 1978; Schneider et al., 1977), and on cognitive functioning (Fillit et al., 1986; Hackman & Galbraith, 1976).

Potential adverse effects and toxicity have, however, been associated with the currently used estrogens. Estrogen hormones are intrinsically lipophilic (Abraham, 1974). The high lipophilicity of these steroids ensures their rapid penetration of biological membranes, including the blood-brain

barrier (BBB), thus enabling access to all cells and organs. Indeed, when these hormones are used therapeutically to specifically target the CNS, the steroids equilibrate among all body tissues due to their high lipophilicity (Pardridge & Meitus, 1979). Moreover, when inside the CNS, there is no mechanism to prevent their redistribution back to the periphery as blood levels of the steroids decline (Davson, 1976; Schanker, 1965). So, even if estrogens can easily gain access to the CNS, they are poorly retained by the brain. As a result, only a fraction of the administered estrogen dose accumulates at or near the site of action in the brain. This property of the estrogens necessitates either frequent dosing or the administration of a depot form of the estrogen in order to maintain therapeutically effective concentrations in the brain (Schanker, 1965; Spona & Schneider, 1977). Both of these treatment strategies lead to sustained increases in peripheral estrogen levels. Since estrogen receptors are present in many peripheral tissues (Walters, 1985), where they mediate a myriad of physiological and pharmacological effects (Murad & Haynes, 1985), it further creates the potential of untoward peripheral toxicities. In fact, constant increases in peripheral tissue exposure to estrogens have been shown in numerous retrospective studies to precipitate various peripheral toxicities, including increased risk of breast and endometrial cancer (Bergkvist et al., 1988; Berkowitz et al., 1985; Ettinger et al., 1988; Persson, 1985; Thomas, 1988), cardiovascular complications (Barrett-Conner et al., 1989; Inman & Vessey, 1968; Kaplan, 1978; Thomas, 1988), and marked interference with hepatic metabolism (Burkman, 1988).

In addition to the peripheral toxicities mentioned above, constant exposure to high levels of E_2 valerate has been shown to induce neuronal degeneration in the hypothalamic arcuate nucleus of both male and female rats (Brawer et al., 1980, 1983). Furthermore, other experimental conditions

which result in constant exposure of the hypothalamus to endogenous estrogens, i.e. constant exposure to illumination can also induce similar arcuate nucleus neuropathological lesion in the rat (Brawer et al., 1983). Conversely, experimental manipulations that essentially eliminate circulating E_2 levels (e.g. ovariectomy) greatly reduce the magnitude of the arcuate nucleus neuropathological responses to constant illumination or senescence in the female rat. Although there is no direct evidence as yet that E_2 is the primary neurotoxic agent responsible for the arcuate lesion, it is noteworthy that this region of the hypothalamus is particularly rich in estrogen receptors as well as E_2-concentrating neurons (Pfaff & Keiner, 1973). Thus, it may be that this region of the hypothalamus is exquisitely susceptible to E_2 in any concentration for prolonged period of time in the adult female rat.

Given the aforementioned evidence for: (i) the central actions and the therapeutic implications of estrogen hormones, and (ii) the major limiting factors associated with the use of currently available estrogen medications, a brain-estrogen delivery system with sustained release of estrogen in that tissue is clearly warranted.

Over the past two decades, the attention and efforts of pharmaceutical research were generally focused on the strategy of improving the efficacy as well as the specificity of pharmaceutical products in order to minimize or even abolish their adverse effects. To fulfill these objectives, novel drug delivery systems have been designed and formulated to achieve rate-controlled and targeted-organ delivery (Bodor, 1987; Bodor et al., 1981, 1987; Bodor & Farag, 1983; Bodor & Simpkins, 1983). This strategy would not only ensure a therapeutic agent preferentially gets to its intended site of action, but it does so at the desired rate in order to satisfy the therapeutic criteria.

A remarkable example is the design of an estradiol-chemical delivery system (E_2-CDS) for the enhanced and sustained release of E_2 in the brain (Bodor et al., 1987). The E_2-CDS exploits the unique architecture of the BBB, which normally excludes a variety of pharmacological agents from the CNS due to their physicochemical properties (Bodor & Brewster, 1983). The E_2-CDS is a redox-based chemical-delivery system and the mechanism of its drug delivery is based upon an interconvertible dihydropyridine $\Leftrightarrow$ pyridinium ion redox carrier (Bodor et al., 1987). After systemic administration of the E_2-CDS, it distributes throughout the body, then, the carrier moiety is quickly oxidized to the corresponding quaternary pyridinium ion (E_2-Q^+) in the brain as well as in peripheral tissues. The charged pyridinium-drug complex is thus locked into the CNS while the same moiety rapidly clears from the periphery because of a 40,000-fold increase in its hydrophilicity. Sustained release of the active, parent drug from the charged pyridinium-drug complex occurs in the brain as a result of enzymatic hydrolysis of the ester linkage. The enzymes involved in cleavage of the ester bond are believed to be non-specific esterases.

Collectively, the ability to preferentially deliver E_2 to the brain, thus sparing non-target peripheral site tissues, should improve the therapeutic index of E_2 by (i) increasing the concentrations and/or residence time of E_2 at its receptor site in the brain and (ii), equally important, decreasing the concentrations and/or residence time of E_2 at the potential peripheral sites of toxicities, thereby decreasing untoward peripheral side effects.

To document the predictive biotransformation behaviors of the E_2-CDS (Bodor et al., 1987), and to further substantiate its effectiveness over the currently used estrogens, extensive and long-term pharmacokinetic and pharmacodynamic studies were conducted. These studies included the

following: (1) development of a reliable, sensitive, and specific method for the simultaneous quantitation of E_2-Q$^+$ and E_2, two metabolites of the E_2-CDS, in a wide variety of rat tissues; (2) determination of the tissue distributions of these metabolites in the whole brain, hypothalamus, anterior pituitary, lung, liver, heart, kidney, uterus, adipose tissues, and plasma of the rat; (3) evaluation of the pharmacodynamic consequences of E_2-CDS following its administration into ovariectomized female rats; and finally (4) assessment of the therapeutic potentials of E_2-CDS in animal models for (i) the menopausal hot flush and (ii) androgen-dependent prostatic hyperplasia.

CHAPTER 2
REVIEW OF THE LITERATURE

This chapter will first present a historical review with respect to the endocrinology/neuroendocrinology of estrogen hormones. This will include some evidence pertinent to their physiological/pharmacological actions in the central nervous system (CNS). Furthermore, since the unique architecture of the brain, the blood-brain barrier (BBB), is of central asset in the design and synthesis of the estrogen-delivery system under investigation, a historical account of the BBB will be discussed. Attempts will be made to identify problems associated with the brain delivery of existing drugs. Finally, example of certain clinical conditions which require the presence of estrogen in the brain as a therapeutic agent will be discussed as well. The purpose of this diverse literature review is to identify and describe the concepts and rationale which were the basis in the design and synthesis of the E_2-CDS, which will be evaluated in detail in later chapters.

Estrogen Hormones

Historical Observations

Ovarian endocrine activity was first demonstrated experimentally by Knauer in 1896 (quoted by Tepperman, 1981). Independently, Sobotta (1896) described the origin of corpus luteum at the same time. Shortly thereafter, Beard (1897) postulated that the corpus luteum might serve a necessary function during pregnancy. The observation by Knauer (1900), who

demonstrated that ovarian transplants prevented uterine atrophy and loss of sexual function in castrated animals, established the hormonal nature of ovarian control of the female reproductive system. Further supporting evidence was provided by Fraenkel (1903), who showed that destruction of the corpora lutea in pregnant rabbits causes abortion. In 1923, Allen and Doisy developed a simple, quantitative bioassay for ovarian extracts based upon changes produced in the vaginal smear of the rat. Two years later, Loewe (1925) reported on a female sex hormone in the blood of various species. Shortly thereafter, Loewe and Lange (1926) discovered a female sex hormone in the urine of menstruating women with the observation that the concentration of the hormone in the urine varied with the phases of the menstrual cycle. These observations set the stage for chemists, who soon isolated independently an active estrogen substance from urine in crystalline form (Butenandt, 1929; Doisy et al., 1929, 1930). In 1935, Doisy et al. (quoted by Tepperman, 1981) characterized the chemical structure of estradiol-17β. However, it was the contributions of Corner and Allen (1929) that firmly established the endocrine function of the corpus luteum. They clearly demonstrated that the abortion following extirpation of the corpora lutea in pregnant rabbits can be prevented by the injection of luteal extracts.

Endocrinological and Biochemical Considerations

Biosynthesis

Estrogens are primarily produced by the follicles and corpus luteum of the ovary and by the placenta during the second and third trimesters of pregnancy. Ovaries secrete estradiol (E_2) and estrone, whereas the placenta produces these and estriol (Ross, 1985; Schwartz, 1981). All these hormones

exhibit estrogenic activity; however, 17 β-E_2 is the major and most potent estrogen produced by the ovaries of most species including the human and the rat. Ovaries are capable of synthesizing cholestrol *de novo* from acetate and subsequently converting it to other steroids, including estrogens, progestins, and androgens (Miller, 1988; Schwartz, 1981) . Data obtained from various enzyme kinetics and steroidal precursor-product relationships indicate the involvement of very large number of distinct enzymes, most are members of the cytochrome P 450 oxidases, in the conversion of cholestrol to active steroid hormones (Miller, 1988; Ross, 1985; Schwartz, 1981). Estradiol is formed from either androstendione or testosterone via an aromatization reaction. This reaction is of central importance in estrogen formation, and it is not limited to the gonads or placenta rather a wide variety of peripheral tissues as well as the CNS can aromatize the A ring of androstendione and testosterone to form estrogens (Canick et al., 1986; Michael et al., 1986).

The formation of estrogens is regulated by the concerted actions of two pituitary gonadotropic hormones: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Richards & Hedin, 1988). FSH influences the growth and maturation of ovarian follicles, whereas LH stimulates the synthesis and secretion of estrogens (Richards & Hedin, 1988). The synthesis and release of LH and FSH are, in turn, regulated by the hypothalamic gonadotropin-releasing hormone (GnRH/LHRH) (Dalkin et al., 1989; Davidson, 1969). Furthermore, feedback effects of E_2 and other gonadal factors on the anterior pituitary and primarily on the hypothalamus influence the synthesis and secretion of LHRH (Kalra & Kalra, 1983, 1989; Plant, 1986; Rosie et al., 1990).

Secretion and transport

The E_2-producing cells in the ovary and corpus luteum do not characteristically prepackage large amounts of steroid hormone for release. Rather, these endocrine tissues store the hormone precursor, cholesterol-ester, in the form of lipid droplets inside the hormone-producing cells (Rossmanith et al., 1990). This indicates that, in these secretory cells, the signal for E_2 release is perhaps tightly coupled to that of estrogen-hormone synthesis. Thus, the newly synthesized hormone will be released into the circulation for transport to target tissues. The signals for release are primarily those of the anterior pituitary tropic factors (LH & FSH). It is believed that E_2 is released in pulsatile fashion and this perhaps is the result of the episodic modulating influence of LH (Rossmanith et al., 1990).

Estradiol hormone, like other steroid hormones, when secreted into the blood, it is primarily transported by carrier proteins. E_2 may be transported by: (a) plasma albumin (60%) with low affinity binding; (b) sex hormone-binding globulin (38%) with high affinity binding; and (c) in free (dialyzable) form (2%) (Moutsatsou & Oakey, 1988; Pardridge, 1988a). The carrier-bound E_2 is biologically inactive and sequestered in plasma while only the free fraction is regarded as biologically active hormone. Recent studies have, however, suggested that the carrier-bound pool of E_2 may also be available for uptake by the target tissues (Pardridge, 1988a). It was suggested that there are two possible mechanisms for the delivery of carrier-bound E_2 to target tissues (Pardridge, 1988a). One mechanism involves interactions between the carrier protein surface and the surface of the organ microcirculation that results in a conformational change about the carrier-binding site and thus enhanced dissociation of E_2 hormone. The second

mechanism involves a receptor-mediated transcytosis of carrier-bound hormone complex in the limiting membrane of the organ microcirculation. However, other investigators have argued against this hypothesis on the grounds that the concept does not readily reconcile with physiological findings (Mendel et al., 1988). That is, the rate of protein-bound hormone dissociation (K_d) is a potentially important factor in the model of "free hormone hypothesis." Thus, a more comprehensive model (equation) which formally takes into account the rate-limiting effects of protein-bound hormone dissociation is more relevant to the experimental observations. The current Pardridge's model (the protein-bound hormone hypothesis) is, however, deficient in this respect.

Metabolism and excretion

The plasma concentration of E_2 at any time represents the net difference between the rate of E_2 secretion and the rate of metabolism in the liver and excretion by the kidneys. There is no apparent limit to the capacity of these organs to metabolize and excrete the E_2 hormone. The liver is the primary organ for metabolizing E_2 hormone (Bolt, 1979). The rate of turnover of E_2 hormone is rather rapid. It has a half-life of about 90 min (von Schoultz et al., 1989). The estrogen is oxidized by the action of a stereospecific dehydrogenase enzyme, using pyridine nucleotides as cofactors, to less active products such as estrone and estriol. The oxidized metabolites are then conjugated as sulfates or glucuronides. The conjugation process renders these metabolites highly water soluble which then are quickly excreted in the urine or bile (Bolt, 1979). These conjugates are biologically inactive; however, the biliary metabolites may undergo further metabolism by action of the intestinal flora. The products are then reabsorbed into the portal circulation

and transported to the liver, a process called enterohepatic circulation (Bolt, 1979). A metabolite of E_2, which comprises at least 20% of the total amount secreted in humans, is the 2-hydroxyl derivative. These metabolites, referred to as catechol estrogens, are shown to have biological activity. The biological activity of the catechol estrogen appears to involve an interaction with catecholamine synthesis, receptors or effectors (Weisz & Crowley, 1986). The conversion to catechol estrogen can occur in a number of tissues, including the CNS (Weisz & Crowley, 1986).

Mechanism of action

Based on the gross anatomical, histological, and biochemical evidence, E_2 is shown to have growth-promoting activities on cells of the target organs such as the anterior pituitary, uterus, vagina, Graafian follicles of the ovary and the mammary gland by increasing protein synthesis and mitotic activity. As early as 1953, Szego and Roberts, seeking an understanding of the mechanism of E_2 action, demonstrated accumulation of ribonucleic acid (RNA) and protein in estrogen-stimulated uterine tissues. Mueller et al. (1958) showed that most of the E_2 effects on RNA and protein synthesis can be blocked by a translation inhibitor (puromycin) and a transcription inhibitor (actinomycin D). These observations led to the proposal that estrogen hormones and steroids in general stimulate or activate the production of nucleic acid templates (mRNAs) and, hence, gene expression (Mueller et al., 1958). Soon after the technological advances of the 1960's and, thus, the availability of tritium-labeled estradiol (^3H-E_2), Jensen and Jacobsen (1962) discovered that the estrogen target tissues (uterus and vagina) selectively concentrated the labeled E_2. These investigators were also the first to demonstrate the binding of E_2 to a specific cytosolic receptor protein (Jensen &

Jacobsen, 1962). Further studies demonstrating the nuclear localization of E_2 receptors (Jensen & DeSombre, 1972) or increases in thymidine incorporation, mRNA polymerase, mRNA synthesis, and protein synthesis (Hamilton, 1968) supported the concept that the primary site of estrogen action in target tissues is within the nuclear genome. Furthermore, voluminous body of available evidence suggests that steroid hormones in general interact with intracellular stereospecific receptors and upon binding the whole receptor-hormone complex translocates into the nucleus (Walters, 1985). The hormone-receptor complex then alters nuclear gene transcription, leading to the production of all classes of RNA before regulating cytoplasmic protein synthesis (Walters, 1985). These actions generally occur with a delay of several hours or days between the arrival of steroids at the target tissue and the first detectable changes in cellular function.

Several recent contributions to the concept of gene expression by steroid hormones have included the following: (1) demonstration of specific DNA sequences that serves as the actual nuclear acceptor site for the steroid receptor (Spelsberg et al., 1984); (2) evidence for the binding of the occupied and/or transformed steroid receptors to DNA components (Gehring & Tomkins, 1974); (3) assessment of gene transcription in purified nuclei, including the demonstration that the estrogen receptor-induced increase in ovalbumin mRNA transcription is not only dose-dependent but also tissue-specific (Taylor & Smith, 1982); and (4) isolation of hormone-induced mRNA sequences and subsequent cloning of their complimentary DNAs (cDNAs) (O'Malley et al., 1979).

In addition to the direct genomic actions (delayed effects) of estrogens described above, some target tissues exhibit very rapid responses to estrogen exposure that are difficult to reconcile with the concept of direct gene

regulation. For instance, the CNS neurones produce diversity of both rapid and delayed neuroendocrine and behavioral effects in response to estrogen exposure (Majewska, 1987). Various studies have suggested that the neuronal plasma membrane may serve as a direct target for the rapid action of estrogens, which may lead to modification of neurotransmitter release or their receptor/effector systems. First, neurophysiological studies on CNS neurons have demonstrated rapid modulation of neuronal excitability, including a brief hyperpolarization and increase in potassium conductance of the postsynaptic membranes of medial amygdala neurones (Nabekura et al., 1986), increase the firing rate of medial preoptic and septal neurones (Kelly et al., 1978), and increase the cerebellar neuronal responsiveness to iontophoretically applied glutamate (Smith et al., 1987) after application of physiological levels of 17 β-E_2. Second, biochemical studies have also provided evidence for the direct actions of estrogens on neuronal membranes. This included an increase in amphetamine-stimulated striatal dopamine release *in vitro* superfusion system with 17 β-E_2 or diethylstilbesterol, but not with 17 α-E_2 (Becker, 1990) as well as *in vivo* microdialysis in freely moving rats (Becker & Beer, 1986). Also, 17 β-E_2 is shown to enhance the responses of adenylate cyclase to biogenic amines in striatal neurons culture (Maus et al., 1989). Finally, morphological experiments have also demonstrated rapid plasma membrane ultrastructural modifications in response to sex steroids application (Garcia-Segura et al., 1987, 1989). Employing freeze-fracture techniques, within 1 min, physiological concentrations of 17 β-E_2 increased the density of exo-endocytotic pits in cerebrocortical and hypothalamic neuronal membranes in culture, which was blocked by estrogen antagonist tamoxifen (Garcia-Segura et al., 1987, 1989). These rapid effects (from seconds to minutes of latency)

with estrogen hormone on neuronal membrane excitability (Garcia-Segura et al., 1987, 1989; Kelly et al., 1978; Nabekura et al., 1986; Smith et al., 1987) occur much too rapidly to be accounted for by new mRNA synthesis and translation into proteins. This has led some investigators to suggest a possible action of gonadal steroids directly on neuronal membrane function/components. Moreover, Pietras and Szego (1979) have reported an increase in cyclic AMP concentrations in uterus within 15 minutes after E_2 treatment. Although actinomycin D (an RNA synthesis inhibitor) can effectively prevent the full expression of long-term E_2 effects on target tissues, the rapid or short-term effects of E_2 seem to be independent of RNA/protein synthesis. These effects are most likely mediated by membrane-associated E_2 receptors (Pietras & Szego, 1979; Towle & Sze, 1983).

Estrogen Receptors

Intracellular/cytosolic receptors

The pioneering studies of Glascock and Hoekstra (1959) and of Jensen and Jacobson (1962) utilizing radiolabeled estrogen demonstrated selective localization and retention of the label in tissues known to be targets for estrogen action. Jensen and Jacobsen (1962) in their studies also demonstrated E_2 binding to a specific cytosolic receptor protein. The application of sucrose-density gradient centrifugation then led to further characterization of the cytosolic estrogen receptor (Toft & Gorski, 1966). Subsequent studies provided further evidence to satisfy the criteria for an E_2 protein receptor. These criteria included the stereospecificity for E_2 binding (Noteboom & Gorski, 1965), saturable or limited number of binding sites (Gorski et al., 1968; Noteboom & Gorski, 1965), size determination by gel filtration

chromatography (Gorski et al., 1968; O'Malley et al., 1969), sucrose gradient analysis (Jensen & DeSombre, 1973; Toft & Gorski, 1966), and sensitivity to heat and proteases but not to nucleases (Gorski et al., 1968; Noteboom & Gorski, 1965; O'Malley et al., 1969).

The question of the subcellular localization of the E_2 receptors was then resolved by a number of experimental criteria. These included subcellular fractionation by differential centrifugation after ^3H-E_2 exposure *in vivo* or *in vitro* (Jensen & Jacobson, 1962; King et al., 1965, Mowles et al., 1971) which provided early evidence for nuclear localization of E_2 receptors. Conversely, E_2 receptors remained in the soluble, high-speed cytosol fraction in tissues which were not previously exposed to the E_2 hormone (Jensen et al., 1968, 1973). Taken together, available data indicate that the unoccupied receptors migrate between both the cytoplasmic and nuclear compartments but are believed to be primarily concentrated in the nuclei in a reversible equilibrium binding state with the nuclear components. Binding of E_2 to the cytosolic receptor results in a transient biologically active occupied transformed receptor. Then the transformed receptor translocates into the nucleus with an enhanced affinity for the nuclear acceptor sites, favoring receptor binding to the acceptor site on the DNA component. This triggers gene expression (changes in mRNA synthesis and modification) and the expression of proteins in the target cell. These effects are generally observed with a delay of several hours or even days between the exposure of the target tissue to E_2 and the first detectable changes in cellular function.

Membrane receptors

Numerous documented reports have emerged to indicate that the rapid actions (from seconds to minutes of latency) of gonadal steroids on

some target tissues may be caused by direct interaction with plasma membrane receptor/effector components (Becker, 1990; Garcia-Segura et al., 1987, 1989; Kelly et al., 1978; Majewska, 1987; Nabekura et al., 1986; Smith et al., 1987; Towle & Sze, 1983). In fact, biochemical studies have demonstrated specific binding sites for sex steroids in synaptosomal plasma membranes prepared from the rat brain (Towle & Sze, 1983). Furthermore, the presence of steroid binding sites have also been demonstrated on plasma membranes of other target tissues as well, including liver (Suyemitsu & Terrayama, 1975), pituitary (Koch et al., 1977), and uterus (Pietras & Szego, 1979). In all these instances, the exact physiological/pharmacological function of the membrane binding sites for steroids has yet to be determined. However, these binding observations are compatible with the rapid non-genomic effects of estrogen, which are not easily accommodated within the genomic model (McEwen et al., 1982, 1984; Majewska, 1987). Collectively, the presence, if real, of these speculative membrane receptors can account for the rapid neurotropic effects of E_2. Furthermore, these receptors may be involved in the modification of CNS neurotransmission.

Estrogen-receptor binding kinetics

Estrogen target tissues, i.e., brain, anterior pituitary, uterus, etc., apparently contain a single, specific estrogen binding component (type I estrogen receptor). The unoccupied receptors migrate between both the cytoplasmic and nuclear components (Walters, 1985). The cytosolic receptors bind E_2 with high affinity such that the steroid-receptor complex remains intact for translocation into the nucleus (Towle & Sze, 1983). In the rat uterus, estrogen-filled binding sites do not undergo detectable degradation over a 24-hr period at temperature up to 30°C (Walters, 1985). Following a

single injection of E_2, the translocation of cytosolic estrogen receptor (ERc) to the nucleus has been reported to be nearly complete within 1 hr in the rat uterus (Jakesz et al., 1983). When ERc was assessed by exchange assay 6 hrs after hormone administration, ERc levels continued to remain very low. However, an increase in nuclear estrogen receptor (ERn) was concomitantly observed following E_2 injection, reaching maximal levels after 1 hr (Jakesz et al., 1983). Following an apparently near quantitative translocation of ERc to the nucleus, ERn concentrations declined to ~ 30% after 6 hrs. However, with repeated injections of estrogen which maintained continuous receptor saturating concentrations of $[^3H]E_2$ over a 6-hr period, conservation of total cellular receptors in both cytoplasmic and nuclear fractions were observed (Jakesz et al., 1983). It is important, however, to note that under continuous steroid exposure qualitative changes in receptor properties (down regulation) occur over time in both cytosol and nuclear compartments. It is thought that the ERc present at 6 hr after estrogen administration originate from a replenished pool of receptors. This replenished pool has been reported to be partially dependent on protein synthesis. However, inhibition of protein synthesis by cycloheximide did not inhibit replenishment after estrogen exposure. Thus, estrogen target tissues, particularly uterus may represent a system in which estrogen receptors replenishment appears to be due entirely to receptor recycling (Jakesz et al., 1983).

Determination of the kinetic binding parameters indicated a high affinity estrogen-binding site (Kd = ~ 10^{-10} M) for brain ERc and a Bmax of ~ 3 fmol/mg protein (Walters, 1985). These binding parameters are similar to estrogen binding kinetics of other tissues. Rate of association with the receptor has been reported to be 4.4×10^5 M^{-1} S^{-1} while rate of dissociation was 2.4×10^5 M^{-1} S^{-1} with $t_{1/2}$ = 80 hrs at 0 to 4°C. Half-life for the clearance of

nuclear estrogen-receptor complexs were estimated to be 2 hrs for 17 β-E_2 (Walters, 1985).

Finally, the maximum biological responses seemed to be determined not by the level of hormone binding to cytosol receptors, but by retention of a small proportion (10 to 15%) of the receptors on a limited number of saturable estrogen receptor-binding sites (acceptor sites) in the nuclear DNA (Walters, 1985). This is supported by the observation of a good correlation between the duration of nuclear occupancy and uterine growth stimulation in a series of short and long acting estrogen and their derivatives.

Role of Estrogen in the Menstrual Cycle

During the normal reproductive life (30 to 40 years), a female menstruates 300 to 500 times (Tepperman, 1981). The menstrual cycle is characterized by monthly rhythmic changes in the secretion pattern of the reproductive hormones and the corresponding changes in the sexual organs as well. The duration of the cycle averages 28 days. The significance of the menstrual cycle are (1) maturation of an ovum and ovulation; (2) preparation of the uterine endometrium for implantation of an embryo; and (3) expression of the secondary sex characteristics associated with the procreative act. The coordination among these events is achieved by precisely timed fluctuation in the production and secretion rates of a number of hormones associated with the hypothalamic-pituitary-ovarian axis. In humans and primates the ovarian E_2 is perhaps the major driving force for the initiation/maintenance of the cycle. This implies that the primary stimulus in triggering the initiation of the preovulatory gonadotropin surge, the central event in the cycle, is caused by the rise in E_2 levels during the late

follicular phase. The dynamic changes of the ovaries have a periodicity of once every 28 days; however, these events are, in turn, regulated by the cyclic changes of pituitary gonadotropins (Ross, 1985; Schwartz, 1981; Yen, 1978). The pattern of gonadotropin secretion is, in turn, maintained by the pulsatile mode of the hypothalamic LHRH secretion (Levine & Ramirez, 1982; Marshal & Kelch, 1986). Finally, the whole cascade of events is regulated by negative and positive feedback effects of ovarian hormones (McCann, 1982; Pohl & Knobil, 1982). In a sense, the dynamic interplay between the brain and pituitary and the dynamics of the ovarian feedback mechanisms govern the reproductive cycle. That is, the momentum gained from one phase of the cycle powers the next phase, and continues on into the next cycle.

Feedback regulation

The hypothalamic-pituitary-ovarian-endometrial axis in females changes markedly with the menstrual cycle. In humans and nonhuman primates, the cyclic pattern of gonadotropin secretion (the LH surge particularly) seems to depend more on ovarian estrogen than neural signals. This is evidenced by the observation that menstrual cycles in primates were maintained with a constant dose of LHRH per pulse with no variation in amplitude or frequency of LHRH administration (Knobil, 1980). These observations suggested that the hypothalamus may only play a permissive role in the control of the menstrual cycle (Knobil, 1980). However, more recent observations in human and nonhuman primates indicate that normal menstrual cyclicity does depend on LHRH pulse parameter that changes in frequency and amplitude during the menstrual cycle (Dalkin et al. 1989; Ferin et al., 1984; Marshal & Kelch, 1986). Studies in animals as well as in humans have shown that LHRH and gonadotropins are secreted in episodic fashion

(Levine & Ramirez, 1982), and the dynamics of the LHRH pulsatile mode is essential for the differential synthesis and release of gonadotropins (Marshal & Kelch, 1986). Furthermore, ovarian hormones concurrently modulate LHRH pulse parameters and, therefore, are important regulators of gonadotropin secretion. For instance, the preovulatory rise in E_2 increases the LH pulse frequency, and since there is a good concordance between LH pulses in plasma and LHRH pulses in hypothalamic portal blood, this indicates that a transient elevation in E_2 levels increases the frequency of LHRH secretion (Marshal & Kelch, 1986). The feedback effects of E_2 not only modulate the release of LHRH from the hypothalamus but also the responsiveness of the pituitary to LHRH signals (Marshal & Kelch, 1986). Therefore, the effects of E_2 on the gonadotropins LH and FSH are coordinated at the levels of both the hypothalamus and pituitary.

Estradiol displays both inhibitory and stimulatory effects on the secretion of these hormones. Inhibitory, or negative feedback effects, are seen during periods of basal LH secretion throughout the menstrual cycle. During the follicular phase, the follicle secretes low levels of E_2, and during the luteal phase, the corpus luteum secretes large quantities of both estrogen and progesterone. The combined effects of these steroids inhibit the secretion of LHRH and consequently reduce the release of pituitary gonadotropins. However, the positive feedback effects, or stimulation, are observed after a transient and progressive increase in the titers of E_2. That is, an increase in serum E_2 levels of 150 pg/ml or greater for 24 to 36 hours during the late follicular phase of the menstrual cycle (Ross, 1981). This occurs in response to the increase in LH pulse frequency which subsequently results in the preovulatory surge of LH. This condition subsequently causes ovulation of the ovum from the Graafian follicle and the formation of a new corpus

luteum. In the absence of gonadal steroids such as following ovariectomy or menopause, the negative feedback effects of estrogens on LHRH and gonadotropins are removed and thus, serum LH and FSH levels increase. From the clinical point of view, constant exposure to estrogens (or maintenance of their elevated levels) prevents the preovulatory surge of LH by exerting a negative feedback mechanism on the hypothalamic-pituitary unit. This strategy represents the primary mechanism by which the E_2 component of contraceptives prevents ovulation. Collectively, the effects of E_2 on the hypothalamic-pituitary unit depend on the exact duration or magnitude (or both) of exposure to the hormone.

Role of Estrogen in the Rat Estrous Cycle

Rats have 4 to 5 days of estrous cycle (Long & Evans, 1922). The estrous cycle, like the menstrual cycle in human and nonhuman primates, represents an extraordinary sequence of events in hormonal and behavioral changes, and it is verified by cyclic changes in vaginal cell morphology (Long & Evans, 1922). The normal cycle consists of one day of estrus, followed by two days of diestrus (I and II), and one day of proestrus. The dynamic relation between the hypothalamic releasing factor, pituitary gonadotropins, and ovarian steroids during the estrous cycle have been reviewed in considerable detail (Kalra & Kalra, 1983). Estrogen hormones are responsible for the maintenance of the estrous cycle, as in the menstrual cycle. On the evening of estrus, the serum E_2 concentrations reach their lowest levels (15 to 20 pg/ml) as the corpus luteum involutes. However, during the days of diestrus as follicular growth and maturation progress under the influence of FSH stimulation, ovaries produce increasing concentrations of estrogens. E_2 levels

peak to 40 to 80 pg/ml on the evening of the proestrus day of the cycle (Butcher et al., 1974; Kalra & Kalra, 1977). E_2 levels then decline rapidly to basal levels on estrus. The production of E_2 by the follicles of the ovaries is controlled via feedback mechanism (Richards & Hedin, 1988).

Feedback regulation

Ovarian cycle of the rat, like the human, is regulated by the pituitary gonadotropins LH and FSH. However, in the rat, the gonadotropins appear to be primarily under the stimulatory influence of the hypothalamic LHRH neuronal activity (Kalra & Kalra, 1980, 1983; McCann, 1982). This is evidenced by the fact that, in ovariectomized steroid-treated rat, the positive feedback effects of estrogen are expressed as a daily signal for mid-afternoon LH hypersecretion which ensues for several days if concentrations of E_2 are maintained at or greater than E_2 levels seen on the proestrus day (Legan et al., 1975). As in the human and primate, the feedback effects of E_2 on the gonadotropin secretion are coordinated at the levels of both the hypothalamus and the anterior pituitary gland (Kalra & Kalra, 1983, 1989; plant, 1986).

In the rat estrous cycle, E_2 also exhibits both inhibitory and stimulatory effects on the hypothalamic-pituitary unit. The inhibitory, negative feedback, effects are observed during periods of basal LH and FSH secretion throughout the estrous cycle or after chronic exposure of ovariectomized rats to E_2. The inhibitory effects of E_2 are exerted within the hypothalamus (the medial basal and medial preoptic areas) and/or the anterior pituitary. Implantation of E_2 in the medial basal hypothalamus (MBH) (Smith & Davidson, 1974) or chronic elevation in serum E_2 levels (Henderson et al., 1977) were shown to suppress LH levels and increase the pituitary responsiveness to LHRH,

suggesting that the primary site of E_2 action is perhaps within the MBH. However, the stimulatory effects, or positive feedback, are observed after a transient increase in follicular estrogen secretion from diestrus II through proestrus or after more than 48 hours of sustained E_2 exposure to ovariectomized rats (Kalra & Kalra, 1983; 1989; Legan et al., 1975). The positive feedback action of E_2 in the estrous cycle is believed to be exerted primarily on the medial preoptic area of the ventral diencephalon since lesioning of this area (Wiegand et al., 1980), or interuption of its connections to the MBH (Halasz & Gorski, 1967), or implantation of antiestrogen clomiphene into this region (Docke et al., 1989) completely prevent the LH surge and ovulation in the rat. The preovulatory rise of E_2 increases the hypothalamic LHRH mRNA (Rosie et al., 1990), the LHRH pulse frequency and pulse amplitude at mid-cycle (Dalkin et al., 1989; Marshal & Kelch, 1986). However, at the pituitary level E_2 increases the responsiveness of the gonadotrophs to LHRH on the afternoon of proestrus. This allows a priming effect and thus augments the response of the pituitary to subsequent LHRH messages. The increase in pituitary responsiveness must precede the hypothalamic LHRH message for the LH surge to occur.

Blood-Brain Barrier

Historical Overview

The blood-brain barrier is vital to brain function. Neurons are extremely sensitive to the ratio of the concentrations of ions across their plasma membranes. Furthermore, the concentrations of excitatory and inhibitory substances in the extracellular neuronal environment must be tightly regulated for optimal brain functioning. Perhaps because of the functional intricacy of neurons, the mammalian CNS acquired this ultrastable cellular environment to perform effectively. This stable extracellular environment is achieved by several morphological and enzymatic components collectively referred to as the blood-brain barrier (BBB). The BBB is not an absolute barrier, but rather a selective and protective barrier (Neuwelt, 1989; Rapoport, 1976; Suckling et al., 1986). Many pharmacological agents are excluded from entering the brain because of the existence of BBB (Bodor & Brewster, 1983; Schanker, 1965).

The concept of barrier was originally postulated by Ehrlich at the end of the 19th Century (Levin, 1977; Pardridge et al., 1975; van Deurs, 1980). When vital dyes, such as trypan blue, were injected into the bloodstream, the dyes penetrated almost all organs of the body but did not enter the brain tissue or the cerebral ventricles (Levin, 1977; Pardridge et al., 1975; van Deurs, 1980). However, when dyes were injected into the cerebral ventricles, brain tissue was stained and the dye did not readily enter the bloodstream. Thus, the lack of staining by the dyes was not an intrinsic property of brain tissue, rather the existence of a dynamic barrier interface between the blood and the brain.

Subsequent studies which utilized different compounds, including drugs and radioactive tracers further substantiated the concept of BBB. Furthermore, it was later discovered that many small molecules were similarly excluded from transport into the brain. This, then, led to the suggestion that BBB is absolute; a concept which was soon thereafter refuted when the nutrient requirements of the brain were elucidated (Davson, 1976).

Ultrastructural studies have shown that there are several differences between the systemic capillaries and the cerebral capillaries which explain their permeability differences. Morphologically, the ultrastructural feature which most distinguishes CNS microvessels is the endothelial cell lining of the these vessels (Brightman, 1977). The morphological features of the brain capillaries were elucidated using horseradish peroxidase (HRP). This relatively small enzyme has a high affinity for radiopaque substances, such as osmium tetroxide which can be visualized, and it does not cross cerebral capillaries (Brightman, 1977). When introduced directly into the brain, it readily diffused throughout the extracellular space but did not pass between the endothelial cells of cerebral capillaries (Reese & Karnovsky, 1967). So the anatomical basis for the BBB was identified as the endothelial lining of the cerebral capillaries. Unlike systemic endothelial capillaries, the cerebral counterparts are joined by tight junctions (Brightman & Reese, 1969). These tight junctions form a zona occludens and provide, for molecules like HRP, an absolute barrier. Morphologically, these junctions consist of aligned intramembranous ridges and grooves which are in close apposition (Oldendorf, 1977; Shivers, 1979). Two additional morphological features of the cerebral capillaries contribute to the BBB as well: (i) cerebral endothelial cells have a paucity of vesicles and of vesicular transport feature and (ii) the perivascular

astrocytic endfeet seem to be involved in the regulation of nutrients flux and uptake of substances from the circulation (Broadwell & Salcmen, 1981).

In addition to the aforementioned structural features contributing to the BBB, the presence of various enzymes in and around the endothelial cells of cerebral capillaries may play a vital role in limiting and perhaps protecting the brain from a variety of blood-borne substances (Hardebo & Owman, 1980). Thus, the presence of catechol-o-methyltransferase, monoamine oxidase, aromatic amino acid decarboxylase and gamma-aminobutyric acid transaminase in the vicinity of cerebral vasculature may restrict the entry of various blood-borne chemicals, i.e., neurotransmitters/neuromodulators or therapeutic agents into the CNS. Such a protective mechanism against circulating neuroactive substances is essential since optimal CNS function requires a delicate balance between neurotransmitter release, metabolism and uptake in the vicinity of neurons. Finally, the enzymatic component of the BBB may also play a role in excluding some of the lipophilic compounds from the CNS which otherwise might passively diffuse through the barrier.

Regarding the nutrient requirements of the brain, there are numerous specialized carrier transport systems which are localized within the BBB for uptake of nutrients from the circulation (Fenstermacher, 1985; Pardridge, 1981, 1983, 1986, 1987). These include, specific carrier-mediated transport systems for numerous classes of nutrients (Pardridge, 1981, 1983), receptor-mediated transport mechanisms for plasma proteins and peptides (Pardridge, 1986), and plasma protein-mediated transport of protein-bound substances (Pardridge, 1988a, 1988b). These transport systems are localized on the luminal (or blood side) as well as on the antiluminal border (or brain side) of the BBB (Pardridge, 1988a, 1988b), and are characterized by saturability and specificity. These transport mechanisms of the BBB, therefore, provide

means for bidirectional movement of selective molecules. However, with few exceptions, these carrier systems are not involved in transport of chemotherapeutic agents across the BBB (Greig et al., 1987).

Potential Asset to Utilize in the Design of Brain-Specific Drug Delivery

The unique architecture of the BBB allows only the transport of compounds either by specific transport systems or by simple diffusion directly through cell membranes if they are to gain access to the brain parenchyma and extracellular spaces. Therapeutic agents are no exception. They can access the brain through either of these routes. Furthermore, the bulk transport of materials is limited due to the sealing of endothelial gap junctions and the lack of vesicular transport system in the cerebral capillaries. As a result, most drugs that enter the CNS must do so by passive diffusion through the phospholipid cellular matrix of capillary endothelial cells. The lipophilicity of drugs, defined by their octanol-water partition coefficient, correlates with their ability to penetrate the BBB for several classes of drugs, including narcotics (Oldendorf et al.,1972), barbiturates (Levin, 1980) and β-receptor antagonists (Cruickshank et al., 1979). Furthermore, drugs which cannot penetrate the BBB can gain access to limited areas of the brain around the circumventricular organs. Collectively, the CNS has evolved mechanisms to protect itself by excluding hydrophilic (polar substances) and other compounds which may be harmful to its optimal functioning. Unfortunately, this barrier mechanism impedes the delivery and transport of many potentially useful therapeutic agents to the CNS, thus severely complicating the effective treatment of brain diseases.

A strategy that could achieve an improved delivery of drugs to the brain with sustained release in that tissue would be of great advantage. A general approach to increase brain concentration of drugs and thus, their effectiveness has been the design of lipid soluble prodrug from water soluble drugs (Bodor, 1981, 1985; Bodor & Kaminski, 1987; Sinkula & Yalkowsky, 1975; Stella, 1975). Prodrugs are pharmacological agents which have been transiently modified to improve their lipophilicity as well as to hinder their rapid metabolic inactivation via enzymes. Ideally, the prodrug is biologically inactive but reverts to the active, parent drug *in vivo* at, or around, the site of action. This transformation can be mediated by an enzyme or may occur chemically as a result of designed instability in the structure of the prodrug. The purpose of prodrug modification is to increase the concentration of the active drug at or near its site of action, thereby increasing its potency/efficacy. By temporary masking the polar groups of a drug, the lipophilicity of the drug is increased and thus, its ability to enter the brain parenchyma is enhanced. Once in the brain, hydrolysis of the masking groups will release the active drug.

Nevertheless, potential problems are associated with the prodrug approach (Gorrod, 1980). For instance, by increasing the lipophilicity of a drug via the prodrug approach, it may not only improve its diffusion through the BBB to gain access to the CNS, but also ensures the uptake of the compound into all other tissues and thus, exposure to a greater drug burden. This is a major limiting factor in the use of prodrugs, especially those with cytotoxicity, i.e. antineoplastic agents, or those with broad spectrum of peripheral site of actions such as steroids. Furthermore, even if enhanced CNS delivery/uptake is achieved via the prodrug approach, the efflux of the drug

is concurrently enhanced from the CNS. This results in poor retention and minimal or no improvement in the biological half-life of the drug.

To overcome the problem of potential general toxicity associated with enhanced lipophilicity of prodrugs, novel redox-based chemical delivery system (CDS) for drugs has been designed which exploits the unique architecture of the CNS BBB (Bodor, 1987; Bodor & Farag, 1983, 1984; Bodor & Simpkins, 1983; Bodor et al., 1981, 1987, 1988). By definition, a CDS is a biologically inert molecule which requires several chemical conversions leading to the active, parent drug at or near the site of action (Bodor, 1987; Bodor & Brewster, 1983). The multiple, facile chemical conversions may lead to (a) selectivity in drug delivery; (b) improve the drug half-life; and (c) decrease the toxicity of the drug. The redox-based CDS utilizes a carrier molecule that can exist as a lipid soluble (in the reduced state) or water soluble (in the oxidized state). The mechanism of its drug delivery is based upon an interconvertible dihydropyridine $\Leftrightarrow$ pyridinium ion carrier (Bodor, 1987). In this brain-specific CDS, the lipoidal dihydropyridine moiety is attached to the drug, thus increasing its lipid solubility and thereby enhancing its permeability through the BBB. The reduced dihydropyridine can be oxidized, after its administration, to the pyridinium ion in the brain as well as in the periphery including systemic circulation. The charged pyridinium-drug complex is thus locked into the brain while the same moiety rapidly clears from the periphery by renal or biliary processes due to its increased hydrophilicity. Sustained release of the active, parent drug from the charged pyridinium-drug complex occurs in the brain as a result of the enzymatic hydrolysis of the ester (or amide, etc.) linkage between the drug and the pyridinium moiety.

Collectively, the ability to preferentially deliver and sustain the release of a drug in the brain, thus sparing non-target site tissues, should improve the therapeutic index of the drug by (i) increasing the concentrations and/or residence time of the drug at its receptor site in the brain and (ii), equally important, decreasing the concentrations and/or residence time of the drug at the potential peripheral sites of toxicities, thereby decreasing its untoward side effects. Furthermore, this approach may be potentially advantageous in the treatment of brain diseases by virtue of the need for lower or less frequent doses of the drug.

This redox-based CDS has been applied successfully to brain-specific delivery of a wide variety of therapeutic agents, including phenylethylamine (Bodor et al., 1981; Bodor & Farag, 1983), dopamine (Bodor & Simpkins, 1983; Simpkins & Bodor, 1985), gamma-aminobutyric acid (Anderson et al., 1987b), β-adrenergic blocking agents (Bodor et al., 1988), antitumor drugs (Bodor & Brewster, 1983), antiviral agents and antibiotics (Bodor & Brewster, 1983), testosterone (Bodor & Farag, 1984), estradiol (Bodor et al., 1987), and norethindrone (Brewster et al., 1987).

The application of this redox-based CDS to estrogens, particularly E_2 (Bodor et al., 1987), has important clinical and research implications since the hormone plays major role in the reproductive and nonreproductive functions by influencing the brain. Estrogens are intrinsically lipophilic and readily enter the CNS; however, when inside the CNS, there is no mechanism to prevent their redistribution back to the periphery and thus exhibit poor retention. Furthermore, because of their inherent lipophilicity, estrogens equilibrate among all body tissues. This property of the steroid necessitates either frequent dosing or the administration of a depot form of the estrogen in order to maintain therapeutically effective concentrations in

the brain. Both of these treatment strategies lead to sustained increases in peripheral estrogen levels. However, when estrogen is attached to the dihydropyridine carrier, the E_2-CDS enhances brain-specific delivery of estrogen by (i) locking the estrogen into the brain following the oxidation of E_2-CDS to the charged pyridinium moiety (E_2-Q+); and (ii) enhancing the rate of elimination of the lipoidal estrogen, in the inactive E_2-Q+ form, from the periphery following its oxidation to the charged, more hydrophilic compound.

Therapies Aimed at Targeting/Enhancing Brain Estradiol Levels

Fertility Regulation

Fertility control may be achieved by a wide variety of mechanical, surgical, and chemical methods. The chemical (steroidal) methods of fertility control was first introduced by Pincus and Chang (quoted by Tepperman, 1981) and since then it has had important repercussions on population growth. Commonly used steroid contraceptives consist of synthetic estrogens in combination with progestins. When given at pharmacological doses and/or constant exposure, E_2 inhibits (via negative-feedback mechanism) the secretion of gonadotropin hormone-releasing hormone (GnRH) from the hypothalamus (Kalra & Kalra, 1983, 1989; Plant, 1986) and hence, of gonadotropins (LH and FSH) from the anterior pituitary (Kalra & Kalra, 1989). The inhibition of the hypothalamic-pituitary-ovarian axis prevents follicular development and therefore ovulation (Briggs, 1976). However, the use of oral contraceptives has been associated with many adverse metabolic changes, including increased risk of coronary atherosclerosis, myocardial infarction in

smokers, liver tumors, hypertension, and changes in glucose metabolism that appear to be estrogen related (Drill & Calhoun, 1972; Firsch & Frank, 1977; Fotherby, 1985; Inman et al., 1970; Kaplan, 1978; Mays et al., 1976; Thomas, 1988). To reduce the magnitude and the spectrum of these dose-related adverse effects of estrogen thus would be to reduce the dose (Bottiger et al., 1980) and/or to use a more natural hormone (Ottosson, 1984). Given the combined decrease in the contraceptive components, there is the possibility that suppression of the hypothalamic-pituitary-ovarian function may not be as effective as with higher dose formulations. Therefore, preferential brain delivery of E_2 with the CDS may provide an effective, long-acting contraceptive by virtue of sustained release of E_2 in that tissue. Furthermore, the adverse peripheral effects associated with the currently used contraceptive steroids may be avoided by lowering the dose or the frequency of ingestion.

Menopausal Syndrome

The cessation of menses, menopause, near the age of 50 is the result of the decreasing production of ovarian estrogens/progestins (Notelovitz, 1986). This loss of ovarian hormones in 75% to 85% of women leads to a number of brain-mediated steroid-withdrawal symptoms (Casper & Yen, 1985; Lauritzen, 1973; Yen, 1977), the most frequent being hot flushes (Clayden et al., 1974; Meldrum et al., 1979). These (patho)physiological alterations appear to be the result of autonomic discharge which causes peripheral vasodilation and heat loss (Nesheim & Saetre, 1982). Replacement therapy with estrogens and/or progestins has been shown to be effective in most menopausal patients in alleviating the symptoms of the disease (Campbell & Whitehead, 1977; Huppert, 1987; Upton, 1984). However, numerous retrospective studies

indicated an increased risk of peripheral toxicities, including the risk of breast and endometrial cancer (Bergkvist et al., 1988; Berkowitz et al., 1985; Ettinger et al., 1988; Persson, 1985; Trapido et al., 1984), cardiovascular morbidity (Barrett-Conner et al., 1989; Kaplan, 1978; Thomas, 1988), and interference with hepatic metabolism (Burkman, 1988). These adverse effects of estrogens are dose dependent. Currently used estrogens are administered either in frequent doses, or as a depot form, in order to maintain therapeutically effective levels in the brain. Both of these treatment strategies lead to sustained increases in peripheral estrogen levels and thus peripheral toxicities. Since currently employed estrogen therapy is contraindicated in many postmenopausal women, and in as much as some women do not respond to the existing steroid medications, the brain-enhanced E_2-CDS with sustained release of E_2 may be more effective in the treatment of menopausal symptoms by providing sufficient E_2 to the brain while avoiding peripheral toxicities.

Prostatic Cancer

The primary objective of hormone therapy in prostate cancer patients is to induce an effective androgen suppression, thus abolishing the growth promoting effects of androgens on the diseased prostate (Brendler, 1988; Cabot, 1896; Huggins & Hodges, 1941; Isaacs et al., 1983; Moore, 1944; White, 1895). Currently a variety of surgical and therapeutic means for inhibiting androgen production (in the testis) or blocking androgen action (in the prostate) are being used. These include castration, high-dose estrogen therapy, GnRH analogues, and antiandrogens (Santen & English, 1989). Castration or high-dose estrogen therapy remain, however, as the treatment

of choice for the endocrine-dependent management of prostatic cancer (van Steenbrugge et al., 1988). Both treatments are reported to be equally effective in (i) suppressing the circulating testosterone levels (Carlstrom et al., 1989); and (ii) controlling the symptoms of advanced prostatic cancer in 70-80% of patients (Klein, 1979). In contrast to castration, high-dose estrogen therapy inhibits (via negative feedback mechanism) the hypothalamic-pituitary-gonadal function leading to chemical castration. However, high-dose estrogen therapy has been shown to cause severe cardiovascular complications in patients (Henriksson & Edhag, 1986) due to the alterations in liver metabolism (von Schoultz et al., 1989). The E_2-CDS may be potentially useful in the treatment of androgen-dependent prostatic cancer by the virtue of its sustained suppression of the hypothalamic-pituitary-testis function leading to chemical castration and thus regression of the tumorous tissue.

Body Weight Regulation

Food intake and body weight may vary during the estrous cycle of the rat (Tarttelin & Gorski, 1971) and the menstrual cycle of primates (Czaja, 1978) including women (Pliner & Fleming, 1983). A consistent observation is that food intake and body weight decrease during the follicular phase of the ovarian cycle when circulating E_2 levels increase. Conversely, food intake and body weight increase during the luteal phase of the ovarian cycle when E_2 levels decline and progesterone levels elevate.

Although relatively few studies have evaluated the potential modulatory effects of E_2 on body weight regulation in human subjects, the available data support a suppressive role of E_2 in appetite and body weight. Morton et al. (1953) in a study involving menstruating women with

premenstrual syndrome (PMS) reported 37% had a craving for sweets during the luteal phase of their menstrual cycles. Increased appetite was reported to be a frequent PMS symptom in 45 women examined by Fortin et al. (as reported by Smith & Sauder, 1969). Pliner and Fleming (1983) studied 34 women and observed that the decreased food intake during the follicular phase was associated with significant weight loss and that the increased food intake during the luteal phase was concurrent with weight gain. Collectively, endogenous estrogen in women has consistently been shown, albeit subtle, to have a suppressive effect on food intake and body weight.

The potential feasibility of E_2-CDS to reduce body weight has been extensively evaluated in the rat (Estes et al., 1988; Simpkins et al., 1988, 1989a,b). Interestingly, a separation between the effects of E_2-CDS on body weight and food intake has been demonstrated. That is, despite weight reduction, no consistent reduction in food intake has been observed, indicating that mechanisms other than reduced appetite are responsible for the weight loss. Collectively, available data indicate that the E_2-CDS chronically suppresses body weight following a single administration in the rat. Furthermore, the compensatory hyperphasia in response to the weight loss is prevented by the E_2-CDS.

Libido/Sexual Dysfunction

Certain regions of the hypothalamus have been identified to be involved in the central integration of sexual behaviors (Christensen & Clemens, 1974). The first direct evidence that sex steroids influence the central structures associated with mediating and integrating sexual behaviors was presented when Harris implanted estrogen directly into the cat's

hypothalamus. Furthermore, hypothalamic lesions prevent sexual behavior in animals, even in the presence of adequate estrogen (Beyer et al., 1976).

Male sexual behaviors are composed of two distinct components: (1) proception, or the awareness and pursuit of a receptive female and mounting to achieve intromission, and (2) comsumation, or penile erection, intromission and eventual ejaculation (Davidson, 1972). It is believed that the expression of the proceptive components are dependent upon the aromatization of testosterone to E_2 in the brain, particularly in the regions of the preoptic area of the hypothalamus and the amygdala (Beyer et al., 1976; MacLusky et al., 1984). When E_2 was implanted into the preoptic area of the hypothalamus, it effectively restored mounting and intromissions in the castrated rat (Beyer et al., 1976). Other studies have shown that the full restoration of masculine sexual behaviors in castrated rats require E_2 action in the brain and dihydrotestosterone in the peripheral tissues (Lisk & Greenwald, 1983). Given the aforementioned evidence regarding brain mediation of sexual behaviors by E_2, the E_2-CDS may be potentially useful in the treatment of sexual dysfunctions or psychological impotence that are not caused by deficits in peripheral androgen-responsive tissue.

Disorders of Depression

It is generally believed that the underlying mechanism(s) of major mood disorders (mania and depression) may include abnormal functions of monoamine transmission (Bunney & Garland, 1982; Leonard & Kuschinsky, 1982). Pharmacological evidence suggests that mania is the result of hyperactivity while depression is due to hypoactivity of monoamines. Depression (unipolar and bipolar) respond well to antidepressant drugs, such

as tricyclic antidepressants or monoamine oxidase inhibitors, which inhibit the monoamines re-uptake or their respective metabolism (Bunney & Garland, 1982; Leonard & Kuschinsky, 1982).

The biological basis for the involvement of estrogens in depression comes from two gynecological problems: premenstrual and postmenopausal syndromes. The premenstrual syndrome (PMS) refers to the various mood changes in relation to the menstrual cycle that are experienced by a large population of fertile age women. When the relationship between symptom development and normal variations during the menstrual cycle was examined (Backstrom et al., 1985), a consistent observation was that there were very few negative symptoms, rather an increased sense of well being during the preovulatory E_2 peak of the menstrual cycle. However, the maximum degree of symptoms or mood changes occurred during the luteal phase of the menstrual cycle when progesterone levels are increased. Clinical studies as early as in 1932 (Bowman & Bender) suggested a possible therapeutic role of estrogen in the treatment of depression. More recently, Klaiber et al. (1979) reported on a double-blind study performed to assess the therapeutic efficacy of estrogen in the treatment of severely depressed women. The estrogen treatment significantly decreased the degree of symptoms compared with placebo (Klaiber et al., 1979).

Since the 1930's, numerous other clinical studies have provided evidence regarding the influence of estrogens on the well-being and mental performance in postmenopausal women. Hawkinson (1938) reported a significant improvement in menopausal symptoms including depression and anxiety. When subjective indices of moods in ovariectomized women were evaluated in response to estrogen replacement therapy by Rauramo (1975), estrogen treatment resulted in an elevation in moods to that which was

described as being close to normal conditions. A recent study conducted by Gerdis et al. (1982), using a variety of psychometric measures to estimate depression, reported that three weeks estrogen treatment (Premarin) in postmenopausal women significantly improved the symptoms of depression. Furthermore, DeLignieres and Vincens (1982) reported improvement of symptoms of depression, aggression, and anxiety in postmenopausal women that were treated percutaneously with E_2 for three months. In another study (Klaiber et al., 1979), postmenopausal women with primary, recurrent unipolar depression and a history of unsuccessful therapy of their depression were treated with estrogen. The evaluation of their progress by the Hamilton Rating Scale for Depression indicated a dramatic improvement in mean Hamilton scores in some patients (Klaiber et al., 1979). These studies suggested that estrogens may have antidepressant activity in postmenopausal patients.

Another gynecological problem associated with gonadal steroid withdrawal is the postpartum psychosis or depression. During the third trimester of pregnancy, E_2 levels increase to about 10 to 40 ng/ml (~ 1000-fold increase over the follicular phase of the ovarian cycle) while progesterone levels increase to about 100 to 400 ng/ml (~ 100- to 400-fold increase over the follicular phase) (Ross, 1985; Schwartz, 1981). After parturition, gonadal steroid levels fall precipitously and thus, it leads to CNS-steroid withdrawal. Although the etiology of postpartum depression is yet unknown, it is speculated that the decline in gonadal steroid levels after parturition may be the primary factor leading to postpartum depression. In severe cases, it has been reported that administration of estrogen to patients alliveated postpartum depression by suppressing lactation (Yalom et al., 1968). Perhaps, since estrogen hormones influence a variety of CNS functions, by modifying

central neurotransmitters/neuromodulators levels involved in mood, or enzymes necessary for their synthesis, or their receptor/effector systems, estrogen therapy may very well benefit the postpartum depressed patients (Luine et al., 1975; Maggi & Perez, 1985). Thus, there is reason to suggest the notion that estrogen therapy with the E_2-CDS may be useful for the treatment of postpartum depression.

Various biochemical studies in the rat, examining the effect of estrogens on biogenic amines and their enzymes, strongly support the behavioral and symptomatic improvements which are observed in postmenopausal patients. Estrogens are reported to inhibit the re-uptake of norepinephrine in the rat brain (Luine et al., 1975) and decrease the activity of monoamine oxidase (Holzbauer & Youdim, 1973; Luine et al., 1975). Additionally, the activity of monoamine oxidase (MAO) in plasma of postmenopausal patients were also reduced in response to estrogen treatment (Klaiber et al., 1976). In fact, MAO activity is lower in women during the preovulatory phase of the menstrual cycle when E_2 levels are highest. In contrast, MAO activity increases during the luteal phase when progesterone levels are highest (Klaiber et al., 1976).

Collectively, the evidence mentioned above support the notion that estrogen therapy may have a rational scientific basis for treatment of depression associated with the decline in endogenous estrogen production. Estrogen treatment of these conditions may influence brain function via effects on a number of neurotransmitter systems involved in mood and other emotional behaviors.

Cognitive Impairment of Menopausal Alzheimer's Type

Since the 1950's, several lines of evidence have accrued to suggest that estrogen hormones may influence certain cognitive functions of the female. First, the decreasing production of endogenous estrogens/progestins after menopause or ovariectomy has been shown to cause changes in cognitive functions, especially in memory, that are prominent among the somatic and behavioral symptoms of menopause (Kopera, 1973; Malleson, 1953; Lauritzen & van Keep, 1978). Second, estrogen treatment of menopausal women with senile dementia of Alzheimer's type is shown to improve both symptomatic treatment and prevents or slows the progression of dementia in these patients (Campbell & Whitehead, 1977; Fedor-Freybergh, 1977; Fillit et al., 1986; Furuhjelm & Fedor-Freybergh, 1976; Hackman & Galbraith, 1976; Honjo et al., 1989; Michael et al., 1970; Sherwin, 1988; Vanhulle & Demol, 1976). Third, numerous biochemical studies have demonstrated that estrogen hormones modulate/enhance cholinergic transmission or activity in regions of the brain that are important for cognitive functions (Eleftheriou & Dobson, 1972; Iramain et al., 1980; Luine et al., 1975, 1980, 1983, 1985; O'Malley et al., 1987). Fourth, estrogen receptors have been identified in nuclei of the basal forebrain structures in the rat (Luine et al., 1975; Morrel et al., 1975; Pfaff & Keiner, 1973), the major loci of cell bodies of cholinergic neurons which innervate the cerebral cortex, limbic system, hippocampus and hypothalamus. These brain regions are believed to be involved in the pathology of Alzheimer's Disease (Coyle et al., 1983). Finally, perhaps the most interesting observation, is that the female:male sex ratio in the prevalence of Alzheimer's Disease is 2:1 (Sulkava et al., 1985).

Taken together the findings reported in the literature suggest a strong link among estrogen, the cholinergic system, and cognitive functioning in women. Using different estrogen preparations and various Memory Tests/instruments to assess cognitive functioning, results indicated that estrogen therapy resulted in improvement in attention span, orientation, memory, mood, and social interaction in a majority of postmenopausal women with cognitive impairments (Fillet et al., 1986; Furuhjelm & Fedor-Freyberg, 1976; Hackman & Galbraith, 1976; Honjo et al., 1989; Vanhulle & Demol, 1976). Furthermore, the improvement in cognitive functioning, in those patients who benefited, was correlated with an increase in circulating concentrations of estrogens (Sherwin, 1988). Additionally, biochemical data obtained from the rat brain further support the involvement of estrogens in cognition. Studies in the rat brain have shown that cholinergic neurons respond to administration of estrogen by (1) increasing the activity of choline acetyltransferase (ChAT, the enzyme that synthesizes acetylcholine) (Eleftheriou & Dobson, 1972; Iramain et al., 1980; Kaufman et al., 1988; Luine et al., 1975, 1980, 1983, 1985;) in the basal forebrain, cortex, hippocampus and hypothalamus; (2) increasing high affinity choline uptake as well as acetylcholine synthesis (O'Malley et al., 1987) in cerebral cortex; and furthermore (3) the responses of cholinergic system to estrogen administration (i.e., enzyme activity) were observed in female but not male rats and positively related to the dose of E_2 and blocked by an estrogen antagonist (Luine et al., 1980, 1983). Finally, E_2 application has been shown to enhance the excitatory actions of Glu, and Glu is known to be essential in long-term potentiation and memory formation (Smith et al., 1987).

Collectively, available data support the role of estrogens in cognitive functioning. However, the hypothesis does not exclude the possibility of the

involvement of other neurotransmitter systems and estrogens in memory. Thus, if estrogens serve to maintain or enhance the activity of cholinergic neurons or serve as a trophic substance which directly or indirectly acts on cholinergic neurons, the idea of enhanced brain exposure to E_2 may be an improvement in cognition. Further, since any therapy which is aimed at treating Alzheimer's type dementia must be chronic in its application to the patient, the sustained release of E_2 from the E_2-CDS is an additional useful benefit. As such, a careful evaluation of the E_2-CDS for the mechanism by which it improves cholinergic function and for its potential application to Alzheimer's Disease patients is clearly warranted.

CHAPTER 3
GENERAL MATERIALS AND METHODS

Drugs and Solutions

Estradiol and Standard Solution

Estradiol-17β (E_2) was purchased from Steraloids, Inc. (Wilton, NH). Standard solutions of E_2 were prepared in ethanol for the *in vitro* studies involving methodology development. Solutions of E_2 were stored at -20°C (stock solution) or 4°C (working standard).

Estradiol-17β incorporated in 2-hydroxypropyl-β-cyclodextrin (HPCD) was provided by the Pharmatec, Inc. (Alachua, FL). Aqueous solution of E_2 was prepared on the day of experiment in 20% HPCD (wt:vol) for *in vivo* injection.

Estradiol-Chemical Delivery System

Estradiol-chemical delivery system, E_2-CDS (3-hydroxy-17β-[[(1-methyl-1,4-dihydropyridine-3-yl)carbonyl] oxy)-estra-1,3,5-(10)-triene) and E_2-Q$^+$ (1-methyl-3-{[(3-hydroxyestra-1,3,5-(10)-triene-17β-yl)oxy]carbonyl} pyridinium iodide) were synthesized as previously reported (Bodor et al., 1987). Briefly, the 3, 17β-dinicotinate ester of E_2 was made by refluxing 17β-E_2 with nicotinoyl chloride or nicotinic anhydride in pyridine. This derivative was selectively hydrolyzed to the 17-monoester of E_2 with potassium bicarbonate in 95% methanol. The monoester of E_2 was then quaternized with methyl

iodide. The delivery system, E_2-CDS, was then prepared by reduction of the obtained E_2-Q+ with $Na_2S_2O_4$. The structure of each intermediate and the final product (E_2-CDS) was confirmed by the nuclear magnetic resonance and elemental analysis: mp 115-130°C. The yields at each synthetic step were 64-94%. Solutions of the E_2-CDS in water containing 20% 2-hydroxypropyl-β-cyclodextrin (wt:vol) were prepared for injection. For E_2-Q+, the working standard solutions were prepared in water/acetone (80:20;vol:vol) to be used in the *in vitro* methodology development.

Estradiol Pellet

Pellets, weighing 100 mg each, were prepared from crystalline E_2 and cholestrol (CHOL) powder. Both E_2 and CHOL were thoroughly mixed in ratio of 0.5% E_2 and 95.5% CHOL and melted in an oil-bath (200°C). Using a heated pasteur pipette, aliquots of the homogeneous mixture were transferred into small molds made of aluminum foil. After cooling, the solidified pellets were unwrapped from the foil and each pellet weight was adjusted to exactly 100 mg. These pellets were used in the experiments described in Chapter 9.

Morphine Pellets

Morphine pellets were compounded in our laboratory, as previously reported (Simpkins et al., 1983), by the method originally described by Gibson and Tingstad (1970). Each pellet contained 75 mg morphine free base (Merk, St. Louis, MO), 37.5 mg microcrystalline cellulose (Avisil, FMC Corporation, Philadelphia, Pa), 0.56 mg Cab-o-sil (Cabot Corporation, Boston, MA) and 1.13 mg magnesium sterate (Fisher Chemical Co., Fair Lawn, NJ).

Animals

The laboratory rat was chosen as the experimental animal for all experiments herewith. Adult male and female Charles River (CD) rats (aged 3-5 months) were purchased from Charles River Breeding Laboratories (Wilmington, MA). These rats weighed 200-250 g upon arrival and were allowed several days to adjust to the animal quarters before conducting an experiment. Animals were housed in a temperature- (24 ± 1°C) and light- (lights on 0500 to 1900 hr daily) controlled room and provided with Purina rat chow and tap water ad libitum. After a 7-day acclimation period, animals were randomly divided into various experimental groups of 7-8 rats per group. This number of rats per group is standard for the field and is based on our estimates of experimental error in response to the drugs that were evaluated in these studies.

In experiments which required surgical procedures, animals were anesthetized with Metofane (Methoxy Flurane, Pitman-Moore Inc., Crossing, NJ). The surgical procedures consisted of subcutaneous (sc) implantation of drugs or steroids, gonadectomy, and atrial cannulation. Female rats were bilaterally ovariectomized (OVX) by a small incision made through the dorsal peritoneal cavity. Male rats were castrated (CAST) by exteriorizing the testicles through a midline ventral incision. For atrial cannulation, in order to facilitate frequent blood sampling from unrestrained animals, a small incision in the neck-chest area was made to expose the external jugular vein. A Silastic catheter (i.d. 0.5 mm, o.d. 1 mm) was then positioned into the right atrium via the external jugular vein. This surgical procedure was done under sodium pentobarbital anesthesia, according to the guidelines described

by Steffens (1969). All animals were then monitored for post surgical recovery before conducting an experiment on them.

Two methods were employed for collecting blood samples. In most experiments, animals were killed by decapitation and the trunk blood was collected in heparinized tubes. In studies which required frequent blood sampling, animals equipped with atrial cannula were transferred to special sampling chambers and serial blood samples (1 ml) were removed through the cannula. All blood samples were collected in a room separate from the animal quarters. The blood samples were centrifuged and the plasma separated and stored at -20°C until hormone analysis by the radioimmunoassay (RIA).

Drug Treatment

Steroid Treatment

The E_2-CDS treatments described in this dissertation were only given intravenously (iv). An aqueous solution of E_2-CDS was prepared in 20% HPCD on the day of injection and administered iv (via tail vein), a procedure which required a brief restraint of the rat without anesthesia.

The 17 β-E_2 treatments consisted of either iv administration (Chapters 6 & 7) or sc implantation of E_2 pellet (Chapter 9). Implantation of the E_2 pellet was performed in animals under light metofane anesthesia. It should be pointed out that these pellets did require pre-conditioning (i.e. soaking in PBS) before implantation. After implantation, each E_2 pellet produces a transient high concentration of E_2 in plasma that is followed by a sustained blood E_2 levels for about 2 weeks.

Morphine and Naloxone Treatment

In experiments which examined the effects of E_2-CDS on the tail-skin temperature (Chapter 9), animals were addicted to morphine. Morphine dependency was produced by sc implantation behind the neck region of one pellet containing 75 mg morphine free base. Two days after the first morphine pellet, two additional morphine pellets were sc implanted. This regimen of morphine treatment has been utilized in our laboratory to consistently produce typical symptoms of morphine dependency and tolerance (Simpkins et al., 1983, 1984) as measured by several tests of analgesia and withdrawal (Gibson &Tingstad, 1970; Simpkins et al, 1983, 1984). These morphine pellets produce serum morphine concentrations of 300 ng/ml by one hr after implantation and remain elevated at this level through 48 hrs (Derendorf & Kaltenbach, 1986). Thus, the sustained release of morphine achieved by the pellet is presumed to produce persistent stimulation of opiate receptors utilizing our treatment regimen.

Naloxone HCl from Dupont Pharmaceuticals (Garden City, NJ) was dissolved in saline and administered (0.5 mg/kg b.w.) subcutaneously.

Plasma Hormone Radioimmunoassays

Protein Hormone Assays

Plasma luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH), and prolactin (PRL) concentrations were measured in duplicate by radioimmunoassay (RIA) using NIADDK kits provided by the National Hormone and Pituitary Program (Baltimore, MD). Plasma LH, FSH,

GH, and PRL values are expressed as ng/ml of either the LH-RP-2, FSH-RP-2, GH-RP-2 or the PRL-RP-3 reference standards, respectively. Values for unknown plasma samples were derived from the 10 to 90% (linear inhibition portion) of the respective standard curves. Radioiodinations of the labeled hormones were performed in our laboratory using standard procedures for a chloramine-T iodination with gel filtration chromatography to separate free iodine from hormone-bound iodine.

The ranges of hormone assay detectability were (1) 0.25 to 20 ng/ml for LH in 50 ul; (2) 2.5 to 200 ng/ml for FSH in 100 ul; (3) 2.5 to 320 ng/ml for GH in 25 ul; and (4) 0.25 to 50 ng/ml for PRL in 50 ul of plasma sample. Plasma samples containing undetectable LH, FSH, GH, or PRL were assigned the respective assay sensitivity of 0.25, 2.5, 2.5 and 0.25 ng/ml for these hormones. All samples for each hormone in an experiment were assayed in a single run.

Steroid Hormone Assays

Coat-A-Count Estradiol kits--employing solid-phase [^{125}I]-radioimmunoassay--designed for the quantitative measurement of E_2 in serum were purchased from Diagnostic Products Corporation (Los Angeles, CA). Each kit is equipped with human serum-based standards having E_2 values ranging from 20 to 3600 pg/ml (0.07 to 13.2 nmol/l) (technical information from Diagnostic Products). The cross-reactivity of the E_2 antibody has been reported to be <0.3% for E_2-Q+ at the concentration of 15 ng/ml and higher (Rahimy et al., 1989a). The cross-reactivity for estriol and estrone has been reported to be 0.32% and 1.1%, respectively (technical information from Diagnostic Products).

Coat-A-Count Testosterone kits--employing solid-phase [^{125}I]-radioimmunoassay--purchased from Diagnostic Products were used for the plasma testosterone (T) assay. The RIA sensitivity for the T assay was 0.2 ng/ml. The cross-reactivity of the T antibody to the DHT and E_2 has been reported to be 3.3% and 0.02%, respectively (technical information from Diagnostic Products).

Statistical Analysis

For the experimental data that were normally distributed, the significance of differences among groups were determined by one- or two-way analysis of variance (ANOVA) (Zar, 1974). Where necessary, data were transformed (ln) prior to ANOVA. Subsequent pairwise comparisons were made by Dunnett's or Scheffe's multiple range tests using Statview 512+ program from BrainPower, Inc. (Calabasas, CA) for the MacIntosh computer. Where appropriate, data were subjected to area under the curve (AUC) analysis using the trapezoid method (Tallarida & Murray, 1981) and group means for AUC were subjected to ANOVA and Scheffe's tests. The level of probability for all tests were $p < 0.05$. The specific statistical design employed in each experiment is indicated in the figure legends.

CHAPTER 4
DEVELOPMENT OF AN ANALYTICAL METHOD FOR THE QUANTITATION OF E_2-CDS METABOLITES IN A WIDE VARIETY OF TISSUES IN THE RAT

Introduction

The estradiol-chemical delivery system (E_2-CDS) offers a novel approach to non-invasively enhance brain delivery and sustained release of E_2 (Bodor et al., 1987). The E_2-CDS is a redox-based chemical-delivery system and exploits the unique architecture of the BBB, which normally excludes a variety of pharmacological agents from the brain (Bodor & Brewster, 1983). The mechanism of E_2-CDS drug delivery is based on an interconvertible dihydropyridine ⇔ pyridinium salt carrier. Figure 1 schematically shows the structures and the mechanisms leading to brain-enhanced and sustained release of E_2. Estradiol, when it is chemically attached to the lipoidal carrier, dihydropyridine, its lipophilicity is further increased and thus, the ability to enter the brain is enhanced. After systemic administration of the E_2-CDS, the carrier system is then quickly oxidized to the corresponding quaternary pyridinium salt (E_2-Q+). This charged moiety of the carrier system reduces its rate of exit from the brain, thereby locking a depot of E_2-Q+ into the CNS. Subsequent hydrolysis of the E_2-Q+ with nonspecific esterases provides sustained release of the active species (E_2) in the brain. Since the E_2-Q+ is hydrophilic (40,000-fold greater than E_2-CDS), its elimination rate from the periphery is predictably much faster than from the brain.

Application of the E_2-CDS to biological systems creates a problem concerning the separation and quantitation of tissue levels of the active species, E_2, in the presence of an excess concentration of the inactive conjugated form of the drug, E_2-Q+. Furthermore, pharmacokinetic and pharmacodynamic studies of the E_2-CDS require a reliable and sensitive method for the quantitative analysis of E_2 as well as E_2-Q+ in biological tissues and fluids. Conventional methods of assaying steroids, especially conjugated forms of E_2, are inefficient and extremely time-consuming. (Bonney et al., 1984; Cortes-Gollegos & Gallegos, 1975; Hoffmann, 1983; Paradisi et al., 1980). RIA is the only method which has been used on a large scale for the analysis of steroid hormones in plasma or serum. Previously described methods for measuring steroidal hormones and their conjugates in biological tissues have been severely hindered by lengthy extraction and repeated purification procedures which are required prior to the use of RIA procedure.

Therefore, the objectives of these experiments were to develop a sensitive, specific method that permits a rapid and reliable quantitation of both E_2 and E_2-Q+ in various biological tissues and fluids. This method development was necessitated by the design of the E_2-CDS which exhibits the predictive, multiple, facile enzymatic conversion to the charged quaternary ion (E_2-Q+) and its subsequent hydrolysis to slowly liberate E_2.

Materials and Methods

Initially, several *in vitro* experiments were conducted to optimize the selective extraction, purification and quantitation of both E_2-Q+ and E_2 from a wide variety of biological tissues and fluids. Subsequently, an *in vivo* study was undertaken to assess the reliability and applicability of the *in vitro*

methodology by determining simultaneously the levels of both E_2-Q+ and E_2 in several tissues following administration of E_2-CDS in the rat.

In Vitro Methodology

Specificity of the estradiol antibody for E_2

The presence of the estradiol conjugate (E_2-Q+) in biological tissues could lead to overestimation of tissue E_2 concentrations if E_2-Q+ cross-reacts with the E_2 antibody. Therefore, possible cross-reaction with the E_2-Q+ was examined by adding E_2-Q+ standards to the RIA of E_2 at concentrations of 2, 5, 15, 50, 180, and 360 ng/ml. These doses of E_2-Q+ were 100-fold greater than the corresponding E_2 standards used to generate standard curves in the RIA of E_2.

Selective solvent extraction of steroids from tissues

Tissues including brain, anterior pituitary, kidney, lung, liver, heart, and adipose tissue were dissected from adult male Charles River (CD) rats (Charles River Breeding Laboratories, Wilmington, MA) immediately following decapitation. All tissues were rinsed in ice-cold saline and stripped of surrounding connective tissue and fat, blotted dry on paper then weighed to the nearest 0.1 mg. Tissue samples of known wet weight were then homogenized (using a Brinkman Polytron Homogenizer; Model PT 10/35) at moderate speed (setting at 6 for two 15-second periods) in an appropriate solvent system (depending on the type of tissue and compound of interest to be extracted). The appropriateness of the solvent system was determined by screening various organic solvents for effective extraction and acceptable recoveries of the steroids, with high reproducibility in the assay procedure. Tissue homogenate pools, at a concentration of 100 mg tissue/ml solvent, were prepared as follows: for E_2 extraction from brain, anterior pituitary,

liver, and kidney, 100% methanol was used; for E_2 extraction from lung, heart, and fat, 100% acetone was used. The reason for using different solvents was that methanol extraction yielded high and consistent (low CV, high CC) recovery of E_2 from brain, anterior pituitary, liver, and kidney but resulted in low or inconsistent recovery of E_2 from lung, heart, and adipose tissue. However, using 100% acetone, an acceptable and consistent recovery of E_2 was observed for lung, heart, and adipose tissue. For E_2-Q+ extraction, all tissue homogenate pools were prepared in water/acetone (50:50; v:v).

E_2 recovery estimation. Duplicate aliquots of homogenate, each having a concentration of 100 mg tissue/ml solvent, were spiked with 90, 180, or 360 pg E_2 and vortexed for 1 min. The added steroid was allowed to equilibrate with the homogenate for 30 min at room temperature. The spiked homogenates were then centrifuged for 10 min at 1,500 x g. The supernatant was decanted into a clean test tube and the pellet discarded. For plasma, duplicate 1 ml aliquots of plasma (from male rats) were spiked with the aforementioned doses of E_2 standards, samples were allowed to equilibrate for 30 min, but no subsequent centrifugation was done. Following extraction, E_2 recovery was determined as described below.

E_2-Q+ recovery estimation. The procedure used for E_2-Q+ adhered closely to that described for E_2 recovery. Duplicate aliquots of homogenate were spiked with 150, 300, and 600 pg E_2-Q+ and processed similarly to E_2-spiked homogenates. Serving as a control for the percent of hydrolysis of E_2-Q+, separate duplicate aliquots of homogenates were processed without the addition of E_2-Q+ standards until after the supernatant were isolated. These supernatants were spiked with 300 pg E_2-Q+.

Blanks. Tissue homogenates or plasma pools were extracted to determine residual E_2 concentrations and thereby served as the estimate of hormone background.

Hydrolysis of E_2-Q^+ in various tissue extracts

In preparation for base-catalyzed hydrolysis of E_2-Q^+, the final volume of all E_2-Q^+ extracts, including the control samples, was brought to 900 ul by the addition of 50% water:50% acetone. To each tube containing E_2-Q^+ solution, 100 ul of 10N NaOH were added to make the reaction medium 1N (pH >13). All tubes were vortexed and allowed to reach steady-state equilibrium for 20 min at room temperature. Under the conditions used, a time-course evaluation of the rate of E_2-Q^+ hydrolysis indicated that the steady-state equilibrium was achieved in less than 15 min and thus, longer incubation times did not work better. Also, it was found that the hydrolysis of E_2-Q^+ under basic conditions was maximized in an aqueous/organic solvent (50% water:50% acetone). After the hydrolysis, the pH of the reaction medium was adjusted to a pH in the range of 6 to 8 with NaH_2PO_4 and HCl. This is an optimal pH range for the maintenance of the integrity of the C_{18} columns. Samples with pH outside this range damaged the column sorbents, and the presence of column material in the assay sample interfered with the RIA for E_2.

Solid-phase extraction and separation of E_2 by C_{18} columns

After C_{18} columns were conditioned with 2 column volumes (6 ml) of HPLC grade methanol followed by a column volume wash (3 ml) with distilled water, the samples to be extracted (E_2 and E_2-Q^+ hydrolyzed extracts) were applied to the columns. Approximately 1 to 2 min were allowed for the column adsorption to be completed, then the columns were washed with 2

ml water:acetone (80:20; v:v). The columns were then allowed to air dry for 3 min before samples were eluted. With aqueous samples, the small amount of residual water in the column was removed with either 50-100 ul of HPLC grade hexane or by air-drying for >3 min. For sample elution, 2 aliquots of 500 ul methanol were applied sequentially to each column and the steroid was eluted under vacuum pressure. The eluates were collected separately into glass test tubes in the vacuum manifold and then dried under a stream of nitrogen gas. Methanol was used to elute E_2 from the columns since we observed a column extraction efficiency of 92% with methanol, whereas two other solvents tested were less efficient in eluting E_2 (88% with acetone and 49% with acetonitrile).

Radioimmunoassay of E_2

The dried residues of the E_2 samples were reconstituted in 300 ul of the assay buffer (kit Zero Calibrator; Lot # 10E2Z003; 100 ml) then, after vortexing for 1 min, samples were equilibrated for 30 to 60 min at room temperature. Duplicate 100 ul aliquots of each reconstituted E_2 samples were assayed by RIA.

Calculations

If the mean blank (tissue homogenates that did not contain the E_2 spike) values for an assay were greater than the limits of sensitivity of the standard curve, (i.e. if detectable E_2 was present in the tissue), these values were subtracted from all spiked samples. Also, values calculated from the RIA run were adjusted for the volume of the aliquot taken for the RIA, experimental losses during solvent extraction and chromatography (determined by the addition of internal standard), and the weight of the tissue sample used (for the *in vivo* experiment).

In Vivo Studies

To evaluate the applicability and reliability of this procedure to *in vivo* condition, adult male Charles River (CD) rats received a single intravenous injection (tail vein) of the E_2-CDS at a dose of 1.0 mg/kg body weight or the drug's vehicle, 2-hydroxypropyl-β-cyclodextrin (HPCD). Animals (6 per group) were then killed by decapitation 1, 7, and 14 days after the drug injection and the trunk blood was collected in heparinized tubes. The blood was centrifuged and the plasma separated and stored at -20°C until hormone analyses. Brains were removed, rinsed with ice-cold saline solution and stripped of their pia matter and immediately stored at -80°C until hormone analysis. Plasma and brain samples were each processed and assayed by the method described under the In Vitro Methodology section.

The 1.0 mg/kg dose of E_2-CDS was chosen for investigation in this study for the following reasons: 1) we anticipated that the tissue concentrations of the E_2-CDS metabolites, E_2-Q+ and E_2, would be in a quantitatable range when using this dose and the application of RIA procedure, over the time-course chosen for this study and 2) our previous pharmacological observations indicated that this dose of the E_2-CDS is capable of causing chronic suppression of gonadotropins in castrated rats.

Results

In Vitro Methodology

Cross-reactivity of the E_2 antibody with E_2-Q+

The inhibition of binding of ^{125}I-E_2 to the E_2 antibody caused by E_2 and E_2-Q+ is shown in Figure 2. While E_2 effectively competed for binding with the labeled hormone (IC_{50} = 368 pg/ml; 1.35 pM), E_2-Q+ was ineffective in displacing the ^{125}I-E_2 in the RIA (IC_{50} = 129,000 pg/ml; 326.50 pM). At concentrations of E_2-Q+ of 15 ng/ml and higher, the cross-reactivity of the E_2 antibody for E_2-Q+ was <0.3%. Cross-reactivity of the E_2 antibody for estriol and estrone has been determined to be 0.32% and 1.1%, respectively (technical information from Diagnostic Products).

Recovery of E_2

Recovery of E_2 was assessed by determining the % of each of three doses of E_2 recovered from the brain, liver, kidney, lung, heart, and fat homogenates (Table 1). Due to limitations in the amount of tissue available, only one dose (180 pg/ml) was tested for homogenates of anterior pituitary glands. Recovery of E_2 from each tissue evaluated was found to be dependent upon the organic solvent used in the extraction step. For brain, anterior pituitary, liver and kidney, E_2 extraction with 100% methanol was found to be superior to two other solvents (acetone and acetonitrile) resulting in E_2 recoveries (average of the three doses tested) of 77% for brain, 78% for anterior pituitary, 72% for liver and 71% for kidney (Table 1). Methanol was determined to be a poor solvent for extracting E_2 from lung, heart and fat tissue, but 100% acetone achieved acceptable recovery of E_2 in these three

tissues. The mean recovery of E_2 was 57% for lung, 62% for heart and 64% for fat tissue. The E_2 recovery from plasma was 81%.

Precision of the E_2 extraction-assay method

The precision of the method of estimating E_2 concentrations in a variety of tissues was determined in three ways. First, we determined the coefficient of variation (CV) for quadruplicate samples of each tissue at 3 different E_2 dose levels. Second, we determined the correlation coefficient (CC) of the E_2 dose-RIA response for each tissue. And third, we determined the tissue weight-RIA response for brain samples taken at various times after rats were treated with the E_2-CDS.

The CV for quadruplicate determinations of E_2-spiked tissue homogenates or plasma pools were in the range of 0.8 to 7.9% with the majority of E_2 doses in each tissue showing a CV of less than 3.0% (Table 1).

The CC for the E_2 dose-RIA response was 0.97 or greater for all tissues, indicating that over the E_2 dose-range tested, the procedure accurately estimated the E_2 concentration of the tissue (Table 1; Figure 3, lower panel).

To determine the accuracy of the method in estimating E_2 concentrations in tissues of different weights and hence, to evaluate for tissue components which may interfere with various steps in the E_2 assay procedure, we evaluated tissue wet weights over the range of 2 to 100 mg (Figure 4, upper panel). Brain tissue from rats treated 1 or 7 days before with the E_2-CDS was extracted and assayed for E_2. The inhibition of binding of ^{125}I-E_2 was correlated with the weight of tissue used for both samples taken at day 1 (CC = 0.99) and 7 days (CC = 0.99) after E_2-CDS administration. The parallelism of the observed inhibition curves indicated that the E_2 concentration measured is independent of the weight of tissues used in the

determination. Also, the rightward shift in the tissue weight-RIA response curve at day 7 indicates a decrease in brain E_2 concentrations with increasing time after drug administration.

Recovery of E_2-Q^+

The recovery of E_2-Q^+ is dependent upon the efficiency of three processes: (a) the extraction efficiency of E_2-Q^+ from tissues with water:acetone; (b) the % hydrolysis achieved under basic conditions; and (c) the % recovery of E_2 following its formation from the hydrolysis of E_2-Q^+. The recovery of E_2 was determined as described above and the parameters described below after the adjustment for E_2 recovery. The recovery of E_2-Q^+ was determined as the percent of the spiked concentration of E_2-Q^+ which was assayed. The % hydrolysis of E_2-Q^+ was determined experimentally for each tissue and the extraction efficiency in water:acetone was calculated as the recovery times the reciprocal of the % hydrolysis.

The extraction efficiency of E_2-Q^+ with water:acetone ranged from 65.4% for heart to 80.7% for liver tissue (Table 2). For each tissue evaluated, the % hydrolysis of E_2-Q^+ was the primary factor limiting the recovery of this species. The % hydrolysis ranged from 69% in liver to 37% in adipose tissue (Table 3). For all tissues except fat and plasma, the % hydrolysis was greater than 50% (Table 3).

Precision of the E_2-Q^+ extraction-assay method

The precision of the E_2-Q^+ method was determined using the same parameters used for demonstrating precision of the E_2 method.

The CV for the quadruplicate determinations of E_2-Q^+-spiked tissue homogenates or plasma pools ranged from 0.9 to 7.3% with a majority of the E_2-Q^+ doses for each tissue showing a CV of less than 5.0% (Table 2).

The CC for the E_2-Q+ dose-RIA response was 0.98 or higher for each tissue evaluated (Table 2; Figure 3, upper panel). Thus over the E_2-Q+ dose range tested, the method accurately estimated E_2-Q+ concentrations in each of the tissues.

Increasing brain tissue wet weight from 0.25 to 50 mg caused a highly correlated decrease in binding of ^{125}I-E_2 in the RIA used at day 1 (CC = 0.99), day 7 (CC = 0.99) and day 14 (CC = 0.99) after the treatment of rats with E_2-CDS (Figure 4, lower panel). The inhibition curves caused by increasing brain tissue wet weight from animals at 1, 7 and 14 days posttreatment were parallel and the rightward shift was indicative of the time-dependent reduction in brain E_2-Q+ concentrations (Figure 4, lower panel).

Distribution of E_2 and E_2-Q+ in vivo

Figure 5 shows brain and serum levels of E_2-Q+ (upper panel) and E_2 (lower panel) at various times following administration of the E_2-CDS (1 mg/kg) or the HPCD vehicle (day 0 values). Brain E_2 concentrations were increased to 11.1 ± 1.4 ng/g tissue (mean ± SEM) on day 1 and remained greater than 3.5 ± 1.1 ng/g at day 14 after drug treatment. A small amount of E_2 was detected in brains of HPCD-treated (control) male rats (0.2 ± 0.07 ng/g), a likely result of the aromatization of testosterone in the brain (Michael et al., 1986). Brain concentrations of E_2 exceeded serum levels of the hormone by 39-, 41- and 82-fold at 1, 7 and 14 days, respectively, after E_2-CDS treatment (Figure 5).

E_2-Q+ levels were increased to 200.9 ± 8.8 ng/g brain tissue at 1 day after administration of the E_2-CDS, and E_2-Q+ levels remained elevated to 67.0 ± 17.2 ng/g at 14 days postinjection (Figure 5). The brain to serum ratio for E_2-Q+ was 33, 70 and 294 at 1, 7 and 14 days, respectively.

In brain tissue, E_2-Q+ levels exceeded E_2 levels by 18-, 22- and 19-fold and in plasma E_2-Q+ levels were 6, 13 and 22-fold higher than E_2 at 1, 7 and 14 days, respectively.

Discussion

This novel, but predictable, metabolism of the E_2-CDS presents several problems for the quantitation of E_2-Q+ and E_2, two metabolites of the E_2-CDS. First, since estradiol is active at tissue concentrations of low pg/g, the assay method for the E_2-CDS metabolite, E_2, must be extremely sensitive. Second, since E_2-Q+, the moiety "locked" in the brain, is expected to be present in concentrations much higher than E_2, the assay method must be capable of distinguishing low levels of E_2 in the presence of high concentrations of E_2-Q+. Third, the accuracy of the E_2 determination is dependent upon the stability of E_2-Q+ against hydrolysis (enzymatic or spontaneous) throughout the procedures. However, under the conditions utilized, E_2-Q+ was quite stable. When 300 pg E_2-Q+ were added to tissue homogenates and evaluated for spontaneous hydrolysis throughout the procedures used, the E_2 recovery was below the sensitivity of the assay. Finally, as for any assay method, necessary features are (a) a high recovery of the species of interest; (b) accuracy of the determinations; and (c) reliability of the method through a wide range of hormone concentrations and tissue weights. We have provided evidence for each of the features of the present method for simultaneous determinations of E_2-Q+ and E_2.

The RIA procedure provides the needed sensitive endpoint for the determination of E_2 and E_2-Q+ levels. This RIA for E_2 is sensitive from 0.8 to 1.2 pg E_2/assay tube and exhibits a highly correlated inhibition of ^{125}I-E_2

binding over the range of 20 to 3600 pg/ml. This level of sensitivity is substantially greater than the recently published HPLC methods which report levels of sensitivity of 50 and 10 ng/ml of plasma for E_2 and E_2-Q+, respectively (Mullersman et al., 1988). Additionally, the antibody used was very specific for estradiol, showing a cross-reactivity of <0.3% for E_2-Q+ and has also been described to cross-react with estriol and estrone at the level of 0.3 and 1.1%, respectively. In brief, the RIA described here is sensitive and specific for E_2.

The recovery of E_2 is dependent upon the extraction efficiency of the organic solvent used and the elution efficiency of E_2 loaded on the C_{18} columns. Since column elution of E_2 with 100% methanol was essentially quantitative, the recovery of E_2 was equivalent to the extraction efficiency. Methanol extraction yielded high and consistent (low CV, high CC) recovery of E_2 from brain, anterior pituitary, liver and kidney but resulted in low or inconsistent recovery of E_2 from lung, heart and adipose tissue. However, using 100% acetone, an acceptable and consistent recovery of E_2 was observed for lung, heart and adipose tissue. While the reason for this tissue-specific solvent extraction is not clear, the results indicate that the judicious choice of solvent allows for the reliable estimate of E_2 concentrations in a variety of tissues. Indeed, E_2 was precisely measured in a variety of tissues over a 4-fold change in the concentration of E_2 or over a 50-fold change in the amount of tissue used in the determination (Figures 3 & 4).

The recovery of E_2-Q+ was limited primarily by the percent hydrolysis of the E_2-Q+, since extraction efficiency in water:acetone (50:50; v:v) ranged from 65 to 81% for an individual tissue. We observed that our base-catalyzed hydrolysis of E_2-Q+ yielded values of 54% to 69% for all tissues except fat (37%) and serum (30%). While base hydrolysis is not complete, the % hydrolysis

was consistent for each tissue and the variation in % hydrolysis was low (CV = 0.8% for plasma to 5.0% for brain tissue). Furthermore, other methods of hydrolyzing E_2 conjugates, such as enzyme- or acid-catalyzed hydrolysis, are much less efficient (11% and 0.8% net hydrolysis, respectively) and require 18 to 24 hours to conduct (Bain et al., 1984; Czekala et al., 1981; Saumande & Batra, 1984; Segal et al., 1960) versus 20 min for base-catalyzed hydrolysis under the present conditions.

Analysis of E_2-Q^+ in adipose tissue was complicated by two factors. First, E_2-Q^+ is hydrophilic (due to its charge and polarity) and requires an aqueous solvent for its effective extraction. This requirement creates problems in separating the supernatant from the pellet because of the formation of a superficial layer of lipid above the supernatant phase. Second, extensive loss of E_2 from the supernatant occurs after E_2-Q^+ was hydrolyzed. This is due to the presence of fatty droplets in the reaction medium into which E_2 partitioned from the aqueous supernatant; and therefore, it was not recovered efficiently when the supernatant was transferred onto the C_{18} column. These two conditions reduced the recovery of E_2-Q^+ from adipose tissue.

Analysis of E_2-Q^+ in heart and kidney tissues posed a different problem. After hydrolysis of E_2-Q^+ extracts in 1N NaOH solution, the hydrolyzed supernatants from heart and kidney required less HCl and NaH_2PO_4 than other tissues to adjust the pH to the range of 6 to 8; the optimal pH range for C_{18} column function. Without determining the exact amount of acid and buffer needed to achieve the optimal pH range for each tissue, erroneously high E_2-Q^+ levels were calculated due to contamination of the assay tube by the column sorbent.

E_2-Q+ recovery from plasma samples was low likely because of protein precipitation caused by hydrolysis and subsequent neutralization. Centrifugation was needed to separate the supernatants from precipitates before their application onto the C_{18} columns. E_2 released during the hydrolysis step likely interacted extensively with albumin and sex steroid-binding globulin and was unavailable for column extraction.

The separation of E_2 and E_2-Q+ was achieved by 3 different techniques in the process of extraction and assay of these two products of the E_2-CDS. First, samples were divided and differentially extracted for E_2 (methanol or acetone) and for E_2-Q+ (50% water:50% acetone). Although this procedure, which depends upon the solubilization of the lipophilic E_2 in methanol or acetone and the more hydrophilic E_2-Q+ in water/acetone effectively extracted the intended steroid, separation of the two species was not complete. However, when extracts were loaded onto the C_{18} column and eluted with 100% methanol, only E_2 was preferentially extracted and eluted by more than 92%. Thus, the column chromatography effectively separated the two species. Finally, the low cross-reactivity of the E_2 antibody for E_2-Q+ (<0.3%) ensured that in samples extracted and chromatographed for E_2, virtually no E_2-Q+ was measured. Moreover, analysis of tissue samples treated with the E_2-CDS required various dilutions for each time point which ensured the expected E_2 values to fit an appropriate part of the standard curve (ED_{20} at 800 pg/ml to ED_{80} at 25 pg/ml). Indeed, dilutions which were performed prior to loading the extracts onto the C_{18} column minimized nonspecific interference by E_2-Q+ and lipids. Lipids decrease steroid radioimmunoassay accuracy and reproducibility (Rash et al., 1980).

Application of these methods to brain and plasma samples obtained at various times after treatment with the E_2-CDS revealed that both E_2 and E_2-Q^+ can be quantitated throughout the 14-day time-course of the study. As predicted, based upon previous reports on brain levels of E_2-Q^+ (Bodor et al., 1987; Mullersman et al., 1988), this "locked-in" form of the E_2-CDS reached a 33-fold higher concentration in the brain than plasma by 1 day after treatment with E_2-CDS and these brain-blood ratios increased to 294-fold by 14 days. Brain E_2 concentrations were similarly and dramatically elevated relative to plasma. These observations are consistent with the proposed brain-enhanced delivery of E_2 with the redox-based E_2-CDS and indicate that the observed distribution pattern of E_2-Q^+ and E_2 may explain the long-term pharmacological effects of the E_2-CDS (Anderson et al., 1988a,b; Estes et al., 1987a,b; Simpkins et al., 1986).

In summary, the described technique for the simultaneous measurement of E_2-Q^+ and E_2 is sensitive, reliable, specific and applicable to a wide variety of tissues in the body. The additional feature of rapidity of the method allows for the determination of about 100 samples in one day. Collectively, these characteristics indicate that the described techniques could be applied to the quantiation of other conjugates of steroid hormones.

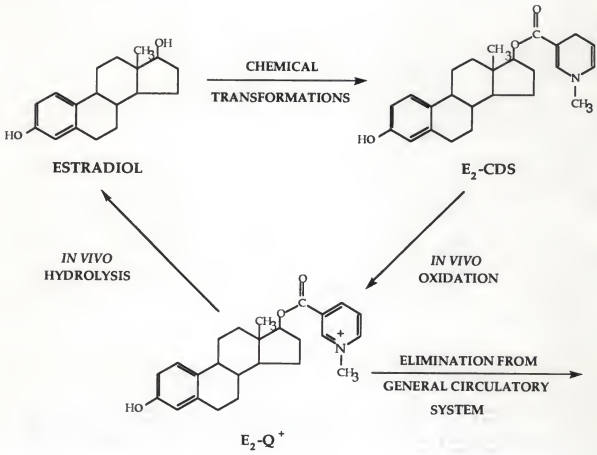


Figure 1. Schematic representation of *in vitro* synthesis and *in vivo* transformation of the estradiol-chemical delivery system (E_2-CDS). E_2-Q+ is the charged quaternary form of the E_2-CDS which is "locked" into the brain and quickly eliminated from the peripheral tissues. Subsequent hydrolysis of E_2-Q+ with non-specific esterases results in sustained and slow release of estradiol in the brain. The trigonelline, carrier moiety, formed upon hydrolysis of E_2-Q+ is non-toxic and is cleared from the brain rather quickly. Although the *in vivo* rate constants for these reactions are unknown among different tissues, the oxidation of E_2-CDS to E_2-Q+ is quite rapid in all tissues analyzed ($t_{1/2}$ = 29 min). However, the rate constant for hydrolytic enzymes may differ among various tissues.

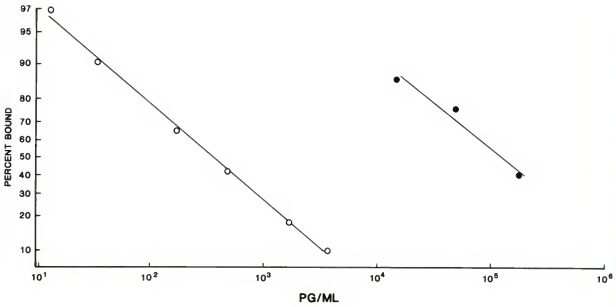


Figure 2. Inhibition of ^{125}I-E_2 binding to an E_2 antibody caused by E_2 (left, open circle) or E_2-Q+ (right, closed circle). Possible cross-reaction with the E_2-Q+ was examined by adding E_2-Q+ standards to the RIA of E_2 at concentrations of 2, 5, 15, 50, 180, and 360 ng/ml. These doses of E_2-Q+ were 100-fold greater than the corresponding E_2 standards used to generate standard curves in the RIA of E_2. Data are expressed on logit-log graph (% bound on ordinate is based on logit = log (percent bound/100 - percent bound)). Cross-reactivity data indicated that E_2 antibody used was specific for E_2 and cross-reacts with E_2-Q+ to <0.3% at concentration of 15 ng/ml and greater.

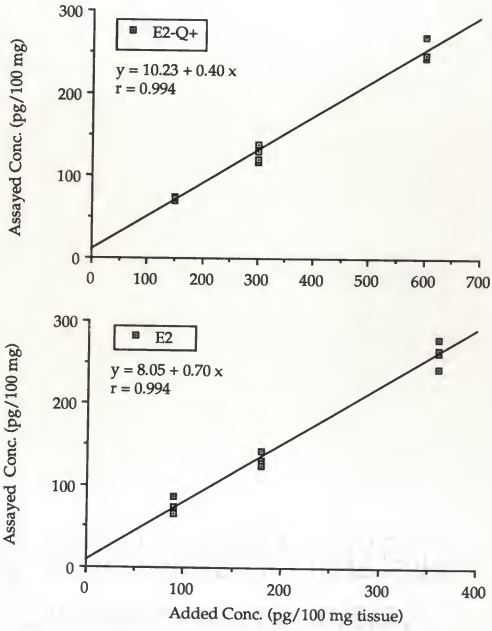


Figure 3. Recovery of known concentrations of E_2-Q+ (upper panel) and E_2 (lower panel) added to brain tissue homogenates prior to extraction. Duplicate aliquots of homogenate were spiked with 150, 300, or 600 pg E_2-Q+ (upper panel) or 90, 180, or 360 pg E_2 (lower panel). After equilibration for 30 min followed by solvent extraction, the spiked homogenates were centrifuged and the supernatant was analyzed for E_2-Q+ or E_2 by the RIA for E_2. The results indicated that the assay method used accurately determines E_2-Q+ and E_2 over a wide range of tissue concentrations.

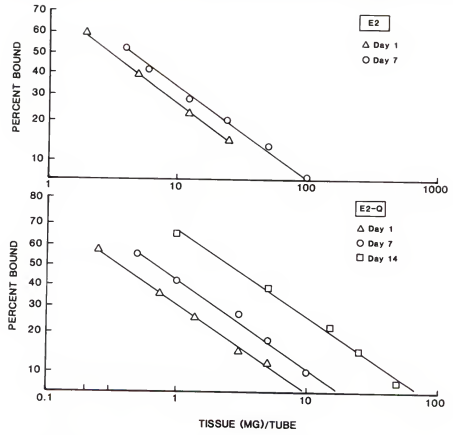


Figure 4. Inhibition of ^{125}I-E_2 binding to an E_2 antibody caused by increasing amounts of brain tissue from rats treated with the estradiol-chemical delivery system (1.0 mg/kg). The upper panel depicts brain tissue wet weights extracted over the range of 2 to 100 mg for E_2 at 1 or 7 days after treatment with E_2-CDS. The lower panel depicts brain tissue extracted over the range of 0.25 to 50 mg for E_2-Q+ at 1, 7 or 14 days after treatment with E_2-CDS. Data are expressed on logit-log graph (% bound on ordinate is based on logit = log (percent bound/100 - percent bound)). The results indicated that the assay method used accurately measures E_2-Q+ and E_2 concentrations over a wide range of tissue weights.

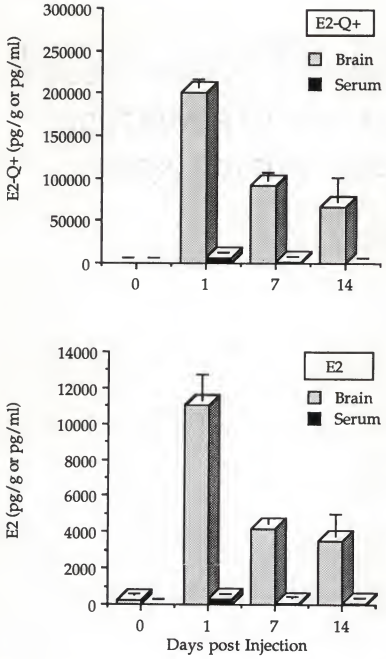


Figure 5. Effects of a single iv dose of the estradiol-chemical delivery system on serum and brain levels of E_2-Q+ (upper panel) and E_2 (lower panel) or the HPCD vehicle (day 0). Each point represents the group mean ± SEM. N = 6 animals per group.

Table 1: Recovery and Precision Determination for Biological Samples Spiked with E_2

Tissue[a]	E_2 Added (pg)	E_2 Assayed[b] (pg)	Recovery (%)	CV[c] (%)	CC[d] (r)
Brain	90	73.9	82.1	7.9	
	180	130.4	72.4	3.3	
	360	268.1	74.5	3.4	0.994
Anter. Pituit.	180	141.0	78.3	1.8	ND[e]
Plasma	90	63.4	70.5	2.5	
	180	151.8	84.3	3.0	
	360	321.3	89.2	2.9	0.997
Kidney	90	71.5	79.4	2.2	
	180	121.8	67.6	1.2	
	360	238.8	66.3	1.8	0.996
Lung	90	52.0	57.8	6.0	
	180	100.0	56.4	3.7	
	360	206.1	57.3	2.4	0.993
Heart	90	52.6	58.5	2.7	
	180	112.6	62.6	2.8	
	360	230.8	64.3	4.4	0.997
Liver	90	69.4	77.1	1.7	
	180	126.5	70.3	1.9	
	360	245.4	68.2	2.5	0.997
Fat	90	66.4	73.8	3.0	
	180	97.4	54.1	0.8	
	360	228.0	63.3	1.1	0.975

a 100 mg of tissue or 1 ml of plasma was used.
b Mean of n = 4 for each dose of E_2 in each tissue.
c CV = coefficient of variation.
d CC = correlation coefficient.
e ND = not determined.

Table 2: Recovery and Precision Determinations for Biological Samples Spiked with E_2-Q+

Tissue[a]	E_2-Q+ Added (pg)	E_2-Q+ Assayed[b] (pg)	EE[c] (%)	Recovery (%)	CV[d] (%)	CC[e] (r)
Brain	150	73.7	74.5	49.2	1.3	
	300	129.6	65.1	43.2	4.6	
	600	249.9	63.1	41.7	4.6	0.994
Plasma	150	47.5	ND	31.7	6.1	
	300	87.8	ND	30.0	2.8	
	600	208.3	ND	34.7	0.9	0.989
Kidney	150	68.4	73.4	45.6	1.0	
	300	137.9	74.0	46.0	3.0	
	600	238.3	64.4	40.0	2.2	0.990
Lung	150	64.0	76.3	42.6	1.0	
	300	131.1	78.2	43.7	2.6	
	600	236.7	71.6	40.0	1.1	0.995
Heart	150	52.6	64.8	35.0	2.6	
	300	104.0	64.1	34.7	4.6	
	600	218.5	67.4	36.4	5.9	0.989
Liver	150	80.4	80.4	53.6	2.2	
	300	164.0	82.0	54.7	6.7	
	600	319.2	79.8	53.2	7.3	0.988
Fat	150	39.8	70.9	26.6	2.1	
	300	78.9	70.2	26.3	2.6	
	600	163.3	72.7	27.2	3.0	0.988

a 100 mg of tissue or 1 ml of plasma was used.
b Mean of n = 4 for each dose of E_2-Q+ in each tissue.
c EE = extraction efficiency.
d CV = coefficient of variation.
e CC = correlation coefficient.
f ND = not determined.

Table 3: Percent Hydrolysis of E_2-Q+ in Supernatants of a Variety of Tissues

Tissue[a]	E_2-Q+ Added (pg)	E_2-Q+ Assayed[b] (pg)	Hydrolysis (%)	CV[c] (%)
Brain	300	198.3	66.1	5.0
Plasma	300	87.8	30.0	2.8
Kidney	300	186.6	62.2	2.9
Lung	300	167.6	55.8	1.8
Heart	300	162.5	54.2	1.7
Liver	300	207.3	69.1	3.5
Fat	300	112.3	37.4	4.1

a 100 mg of tissue or 1 ml of plasma was used.
b Mean of n = 4 for each dose of E_2-Q+ in each tissue.
c CV = coefficient of variation.

CHAPTER 5
DETERMINATION OF THE TISSUE DISTRIBUTION OF E_2-CDS METABOLITES IN MALE RATS

Introduction

Estrogens are intrinsically lipophilic (Abraham, 1974) and readily cross the blood-brain barrier (BBB) to gain access to the central nervous system (CNS). However, when inside the CNS, there is no mechanism to prevent their redistribution back to the periphery as blood levels of the steroid decline (Davson, 1976). Indeed, when these hormones are used therapeutically to specifically target the CNS, the steroids tend to equilibrate among all body tissues due to their high lipophilicity (Pardridge & Meitus, 1979). As a result, only a fraction of the administered dose accumulates at or near the site of action in the brain. This property of the estrogens necessitates, either frequent dosing, or the administration of a depot form of the estrogen to achieve and maintain therapeutically effective concentrations in the brain (Spona & Schneider, 1977). Both of these treatment strategies lead to sustained increases in peripheral estrogen levels.

Furthermore, estrogen receptors are present in many peripheral tissues (Walters, 1985), where they mediate a myriad of physiological and pharmacological effects (Murad & Haynes, 1985; Walters, 1985). This further creates the potential of untoward peripheral side effects (Thomas, 1988). In fact, constant increases in peripheral tissue exposure to estrogens have been shown in numerous studies to precipitate various peripheral toxicities, including risk of breast and endometrial cancer (Hurst & Rock, 1989; Persson,

1985; Thomas, 1988), cardiovascular complications (Barrett-Conner et al., 1989; Drill & Calhoun, 1972; Inman & Vessey, 1968; Kaplan, 1978), and alterations in hepatic metabolism (Burkman, 1988).

Since the brain is the primary site where E_2 exerts its beneficial effects on the estrogen withdrawal symptoms at the menopause (Casper & Yen, 1985; Lauritzen, 1973; Yen, 1977), to inhibit gonadotropin secretion for fertility regulation (Goodman & Knobil, 1981; Kalra & Kalra, 1989; Plant, 1986), to reduce growth of peripheral steroid-dependent tissue tumors such as the prostate (Rao et al., 1988), and to stimulate male and female sexual behaviors (Beyer et al., 1976; Christensen & Clemens, 1974), a brain-enhanced delivery with sustained release of E_2 in that tissue is warranted. The ability to deliver E_2 preferentially to the brain, thus sparing non-target site tissues, should improve the therapeutic index of E_2 by (i) increasing the concentrations and/or residence time of E_2 at its receptor site in the brain and (ii), equally important, decreasing the concentrations and/or residence time of E_2 at the potential peripheral sites of toxicities, thereby decreasing untoward peripheral side effects.

Having established a reliable, specific method for the simultaneous quantitation of E_2-CDS metabolites in various tissues (Chapter 4; Rahimy et al., 1989a), and thus to demonstrate the effectiveness of the E_2-CDS, extensive time-course studies were undertaken to evaluate the tissue distribution of E_2-Q^+ and E_2 in both male and female rats. The objective of this study was to evaluate a general tissue distribution of E_2-Q^+ (the intermediate, oxidized metabolite of the E_2-CDS) and E_2 (the active, parent steroid released upon hydrolysis of the E_2-Q^+) in brain, anterior pituitary, lung, liver, kidney, heart, fat, and plasma following a single iv dose of 1 mg/kg of E_2-CDS in the male rat.

Materials and Methods

Adult, intact male Charles River (CD) rats (225-250 g) received a single iv injection (tail vein) of the E_2-CDS at a dose of 1.0 mg/kg body weight or the drug's vehicle, 2-hydroxypropyl-β-cyclodextrin (HPCD). Rats (6-7 per group) were killed by decapitation 1, 7 or 14 days after the drug administration and the trunk blood was collected in heparinized tubes. The blood was centrifuged and the plasma separated and stored at -20°C until hormone analysis. Tissues (brain, anterior pituitary, lung, liver, kidney, heart, and fat) were dissected immediately following decapitation and stored at -80°C until hormone analysis.

Tissue samples of known wet weight at a concentration of 1 mg/20 µl solvent were processed and assayed by the method described in Chapter 4 (Rahimy et al., 1989a). Tissue homogenates and plasma from HPCD-treated rats were also extracted to determine the residual E_2 concentrations and thereby served as the estimate of hormone background.

Coat-A-Count Estradiol kits--a solid-phase [^{125}I]-radioimmunoassay--designed for the quantitative measurement of E_2 in serum were used for the assay of E_2 in all tissue and plasma samples. Cross-reactivity of the E_2 antibody was determined to be <0.3% for E_2-Q+ at a concentration of 15 ng/ml and higher (Chapter 4). All the purified dried E_2 unknowns were reconstituted in 300 µl of the assay buffer (kit Zero Calibrator) and assayed in duplicate by the RIA. The intra-assay coefficient of variation for E_2 was 1.56% and all samples were determined in two assay runs.

Calculated values obtained from the RIA run were adjusted for the volume of the aliquot taken for the RIA, experimental losses during solvent

extraction and chromatographic separation (using internal standard), and the weight of the tissue sample used.

Results

The results of this experiment are presented in Figures 6-9. Figure 6 shows brain (upper panels) and plasma (lower panels) concentrations of E_2-Q^+ and E_2 at various times following administration of the E_2-CDS. Brain E_2-Q^+ concentrations increased to 318 ± 14 ng/g tissue (mean ± SEM) on day 1, followed by a linear decline to 39 ± 2 ng/g on day 14. This result indicated a reduction in E_2-Q^+ concentration of 46% by 7 days and 88% by 14 days after administration of the E_2-CDS. In contrast, plasma concentrations of E_2-Q^+ increased to 6.1 ± 0.3 ng/ml on day 1, then rapidly decreased by 79% at day 7 and remained at very low levels (0.23 ± 0.03 ng/ml) at day 14 after the E_2-CDS treatment.

Brain concentrations of E_2 increased to 8 ± 0.5 ng/g (day 1) then decreased steadily to 2 ng/g (day 14), indicating a sustained-release behavior from brain E_2-Q^+. In contrast, plasma E_2 concentrations increased to only 0.28 ± 0.1 ng/ml (day 1) and steadily declined thereafter.

Figure 7 shows the time-concentration profiles of E_2-Q^+ (upper panel) and E_2 (lower panel) in liver and fat tissues at various times following administration of the E_2-CDS. Both E_2-Q^+ and E_2 were detected in these tissues throughout the time-course studied. As expected, these tissues showed rapid clearance of E_2-Q^+ as well as E_2. The E_2-Q^+ concentrations decreased from 77 ± 10 ng/g and 71.7 ± 19.5 ng/g (day 1) to 5.4 ± 1.3 ng/g and 1.9 ± 0.5 ng/g (day 14) in liver and fat, respectively. This indicated a reduction

in concentrations of greater than 83% and 87% from 1 to 7 days and 93% to 98% by 14 days after drug administration in liver and fat, respectively.

Similarly, E_2 concentrations in these tissues fell by more than 84% and 80% from day 1 to day 7, and by 14 days after drug administration, the E_2 concentrations decreased by 95% and 90%, respectively.

Figure 8 shows the time-concentration profiles in kidney, heart, lung, and anterior pituitary concentrations of E_2-Q+ (upper panel) and E_2 (lower panel) at various times following administration of the E_2-CDS. The E_2-Q+ concentrations in these tissues initially increased to 1906 ± 131, 1047 ± 106, 748 ± 28, and 407.6 ± 50.6 ng/g in heart, lung, kidney, and anterior pituitary, respectively. These E_2-Q+ levels decreased rapidly by more than 76%, 79%, 74%, and 80% by day 7 in these 4 tissues, respectively. By 14 days after drug administration, E_2-Q+ concentrations decreased by greater than 98% in heart and lung, 96% in kidney, and 93% in anterior pituitary. Despite high initial concentrations of E_2-Q+ in these peripheral tissues, brain levels of E_2-Q+ exceeded E_2-Q+ levels of these tissues by 1.5- to 3-fold at 14 days after administration of the E_2-CDS.

Estradiol concentrations in heart, lung, kidney, and anterior pituitary (Figure 8; lower panel) were similarly elevated on day 1 but decreased rapidly by 67% in heart, 83% in lung, 81% in kidney, and 86% in anterior pituitary by day 7. From day 1 to day 14, the E_2 levels in these tissues decreased by more than 95% of the initial concentrations.

Figure 9 depicts brain (upper panels) and anterior pituitary (lower panels) contents of E_2-Q+ and E_2 at various times after administration of the E_2-CDS. Following a single injection of the E_2-CDS, the brain E_2-Q+ content was 635 ± 28, 340 ± 23, and 77 ± 3.9 ng/brain at 1, 7 and 14 days, respectively.

By contrast, the anterior pituitary content of E_2-Q+ was only 1/260 to 1/170 of that observed in the brain.

Similarly, the brain E_2 content was 15.8 ± 0.9, 10.4 ± 0.8, and 3.2 ± 0.1 ng/brain at 1, 7 and 14 days following administration, respectively, while the anterior pituitary E_2 content was 0.47 ± 0.04, 0.06 ± 0.004, and 0.035 ± 0.006 ng/anterior pituitary at these sampling times. As such, the anterior pituitary E_2 content was only 1/90 to 1/34 of that observed in the brain throughout the time-course studied. Thus, the absolute amounts (contents) of E_2 and E_2-Q+ were many fold higher in the brain even though the anterior pituitary concentrations of E_2 and E_2-Q+ were initially higher than in the brain.

Discussion

The results of this single-dose distribution study demonstrated that both E_2-Q+ and E_2, two metabolites of the E_2-CDS, were present in all tissues analyzed up to 14 days (the last sampling time) after treatment of male rats with the E_2-CDS. Moreover, over the time-course studied, the distribution profiles indicated that: a) regardless of the tissues evaluated, E_2-Q+ levels were many fold higher than E_2 levels at each time point in a particular tissue, indicating a slow rate of hydrolysis of E_2-Q+ to E_2; b) the increased brain/plasma ratios of E_2-Q+ as well as E_2, confirmed that "locking" of the charged moiety, E_2-Q+, into the brain had occurred; and c) E_2-Q+ is retained in the CNS tissue but is rapidly cleared from the peripheral tissues, an observation which is predicted by the inherent physicochemical properties of the delivery system.

Brain-distribution profile revealed that E_2-Q+, the quaternary form of the delivery system, persists in the brain with a half-life of about 8 days, but it

is rapidly cleared from the periphery. This is in accordance with that reported in other studies (Mullersman et al., 1988). The half-life of the lipophilic E_2-CDS in brain tissue is only 29.2 min (Bodor et al., 1987), indicating rapid oxidation of the delivery system to E_2-Q+. From this store of E_2-Q+, E_2 can be slowly released chronically in the brain through nonspecific hydrolysis.

As predicted from the physicochemical properties of E_2-CDS as well as previous reports on brain levels of E_2-Q+ (Boder et al., 1987; Mullersman et al., 1988; Simpkins et al., 1986), this "locked-in" form of the E_2-CDS reached a 52-fold higher concentration in the brain than plasma by day 1 after treatment with the E_2-CDS, and these brain-blood ratios increased to 132-fold at day 7 and to about 170-fold by 14 days. Furthermore, from day 1 through 14, the content of E_2-Q+ in the brain was 6- to 23-times the content of E_2-Q+ in the blood. Thus, a portion of the E_2-Q+ found in plasma may arise from brain stores of the compounds. E_2-Q+ can be cleared from the brain by bulk flow of cerebrospinal fluid (Boder & Brewster, 1983; Schanker, 1965).

Brain E_2 concentrations were similarly elevated relative to plasma. Estradiol achieved a 28-fold higher concentration in the brain than plasma by day 1 and this ratio increased to more than 50-fold at day 7 and remained at 37-fold by 14 days. Additionally, throughout the time-course studied, the brain E_2 content was 3- to 6-times the content of E_2 in the blood. These observations indicated that brain E_2 is continuously produced, and as such the steady-state brain E_2 concentration is dependent on its rate of production from the E_2-Q+ and its rate of elimination from the brain by local metabolism and/or redistribution down a concentration gradient to the plasma. Brain stores of E_2 could contribute to plasma levels through its partitioning to the periphery down a large concentration gradient.

Levels of E_2-Q+ in the anterior pituitary were surprisingly high on day 1 then dropped rapidly to below that of brain levels by day 7 and steadily decreased thereafter throughout the observation period. This initial rise in E_2-Q+ levels may be attributed to increased anterior pituitary uptake of the E_2-CDS followed by its rapid metabolism and clearance. Furthermore, the relative elevation of E_2-Q+ as well as E_2, from day 7 to day 14, in anterior pituitary may be caused by the anatomical relationship between the hypothalamus and anterior pituitary gland. Estradiol released upon the hydrolysis of E_2-Q+, or the E_2-Q+ itself, which is locked into brain, could be delivered directly to the anterior pituitary by the capillary plexus of the hypophyseal portal system. These capillaries in the median eminence lack features of other brain capillaries and hence are not part of the blood-brain barrier (Traystman, 1983). Thus, the median eminence would not be expected to prevent the efflux of E_2-Q+ from the brain, and thus transfer of E_2-Q+ to the anterior pituitary can be expected.

High levels of E_2-Q+ seen in the kidney are likely because this organ is a major site for the elimination of all metabolites of the E_2-CDS. However, the reasons for initial high levels of E_2-Q+ in the lung and heart tissues of the male rat are not clear. We speculate that since these organs receive high blood flow, a substantial amount of the E_2-CDS is delivered to and taken up by these tissues initially. Additionally, despite higher E_2-Q+ levels in heart relative to those of lung, kidney, and anterior pituitary, the heart E_2 concentrations were lower than these 3 tissues. Perhaps, a slow rate of hydrolysis of E_2-Q+, or a slower E_2 metabolism, in heart tissue could contribute to the higher levels of E_2-Q+ and lower levels of E_2 in this tissue.

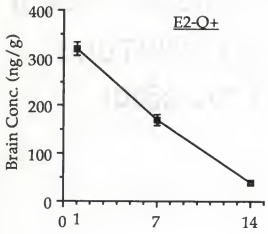
This single-dose pharmacokinetic study supports the previous observations of prolonged pharmacodynamic effects of the E_2-CDS following

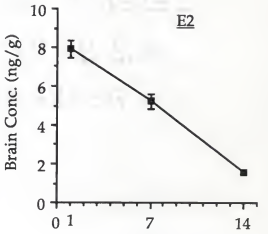
administration of a single dose. LH secretion in castrated male rats was suppressed for greater than 21 days (Estes et al., 1987b; Simpkins et al., 1986), sexual copulatory behavior was stimulated for 28 days (Anderson et al., 1987a), and body weight was suppressed for 36 days (Estes et al., 1988; Simpkins et al., 1988) after doses of E_2-CDS of 1 to 3 mg/kg. These prolonged effects of the E_2-CDS are consistent with the observation here of the accumulation of E_2-Q+, the oxidized form of the delivery system, in the rat brain and its long half-life ($t_{1/2}$ = 8 days) in this tissue. From this store of E_2-Q+, E_2 is released through slow hydrolysis and exhibits a half-life similar to that of E_2-Q+.

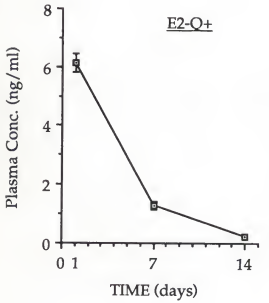
Finally, since we evaluated the tissue distribution of E_2-CDS metabolites in intact male rats, determination of E_2-CDS metabolites in the testes, prostate, and seminal vesicle tissues would have certainly added more valuable informations to the results presented in this chapter. Unfortunately, at the time of experimental investigation, these tissues were not collected for analysis of E_2-CDS metabolites. Certainly, in future studies involving E_2-CDS, these tissues need to be evaluated for E_2-Q+ and E_2 distribution and clearance. Furthermore, the question of blood-testes barrier must be addressed. This would be of great interest to find out whether E_2-Q+ is being "locked" in this tissue similar to CNS tissue or behaves like the rest of peripheral tissues. However, effects of the E_2-CDS on weights of these androgen-responsive tissues were examined and the are presented in Chapter 8.

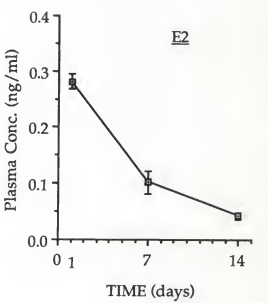
In conclusion, these observations are consistent with the proposed mechanism of the redox-based E_2-CDS and the contribution of the BBB to the chronic retention of the charged, hydrophilic E_2-Q+ in the rat brain. This observed tissue distribution pattern of E_2-Q+ and E_2 may explain the long-term pharmacological effects of the E_2-CDS in the male rat.

Figure 6. Effects of a single iv dose of the E_2-CDS (1.0 mg/kg) on brain (upper panels) and plasma (lower panels) concentrations of E_2-Q+ and E_2 in intact male rats. Intact male rats were injected with a single iv dose of 1.0 mg E_2-CDS/kg bw and killed by decapitation 1, 7, or 14 days after treatment . Whole brain tissue and plasma were processed and assayed for E_2-Q+ and E_2 by the method described in Chapter 4. Each point represents the group mean ± SEM (n = 6-7 rats for each time point).


E2-Q+
Brain Conc. (ng/g)
400
300
200
100
0
0 1
7
14


E2
Brain Conc. (ng/g)
10
8
6
4
2
0
0 1
7
14


E2-Q+
Plasma Conc. (ng/ml)
8
6
4
2
0
0 1
7
14
TIME (days)


E2
Plasma Conc. (ng/ml)
0.4
0.3
0.2
0.1
0.0
0 1
7
14
TIME (days)

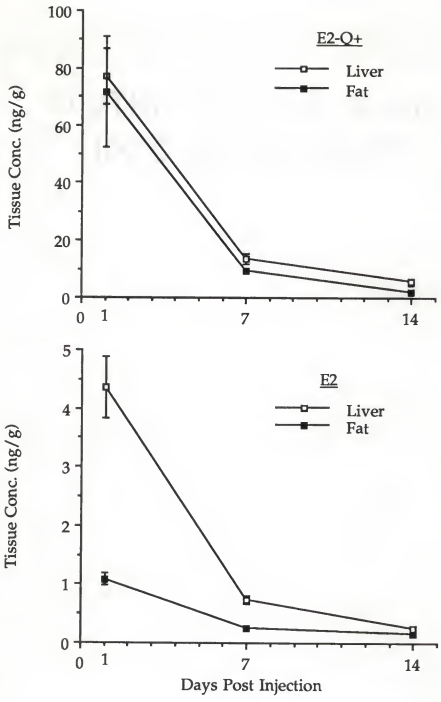


Figure 7. Effects of a single iv dose of the E_2-CDS (1.0 mg/kg) on liver and fat concentrations of E_2-Q+ (upper panels) and E_2 (lower panel) in intact male rats. Intact male rats were injected with a single iv dose of 1.0 mg E_2-CDS/kg bw and killed by decapitation 1, 7, or 14 days after treatment. Each point represents the group mean ± SEM (n = 6-7 rats for each time point).

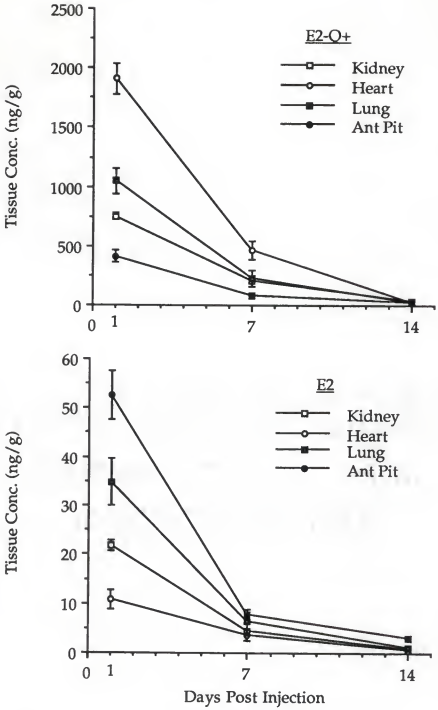
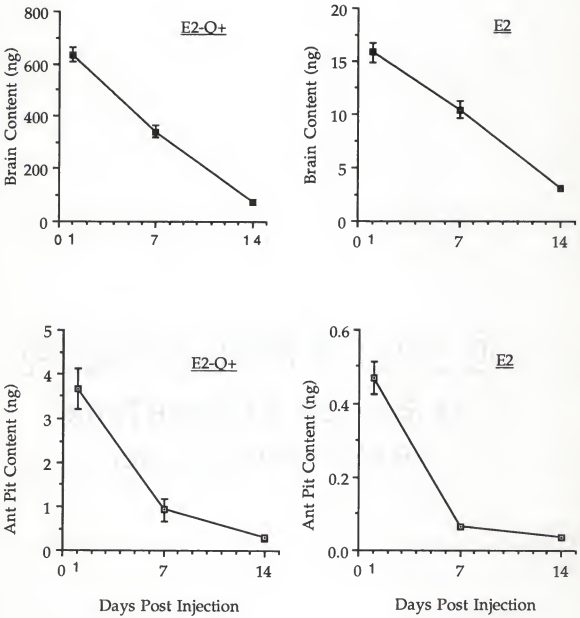


Figure 8. Effects of a single iv dose of the E_2-CDS (1.0 mg/kg) on kidney, heart, lung, and anterior pituitary concentrations of E_2-Q+ (upper panel) and E_2 (lower panel) in intact male rats. Intact male rats were injected with a single iv dose of 1.0 mg E_2-CDS/kg bw and killed 1, 7, or 14 days after treatment. Each point represents the group mean ± SEM (n = 6-7 rats for each time point).

Figure 9. Brain (upper panels) and anterior pituitary (lower panels) contents of the E_2-Q+ and E_2 following a single iv dose of the E_2-CDS (1.0 mg/kg). Intact male rats were injected with a single iv dose of 1.0 mg E_2-CDS/kg bw and killed by decapitation 1, 7, or 14 days after treatment . Whole brain tissue and the anterior pituitary were processed and assayed for E_2-Q+ and E_2 by the method described in Chapter 4. Each point represents the group mean ± SEM (n = 6-7 rats for each time point). These calculations assumed a brain wet weight of 2 grams (based on personal experience and knowledge).


E2-Q+
Brain Content (ng)
800
600
400
200
0
0 1
7
14
E2
Brain Content (ng)
20
15
10
5
0
0 1
7
14
E2-Q+
Ant Pit Content (ng)
5
4
3
2
1
0
0 1
7
14
Days Post Injection
E2
Ant Pit Content (ng)
0.6
0.4
0.2
0.0
0 1
7
14
Days Post Injection

CHAPTER 6
DETERMINATION OF THE TISSUE DISTRIBUTION OF E_2-CDS METABOLITES IN FEMALE RATS

Introduction

Brain-enhanced delivery to and the sustained release of E_2 in the brain may be potentially useful for the effective treatments of vasomotor hot flushes, prostatic adenocarcinoma, and fertility regulation. The natural estrogen, 17 β-E_2, administered as either a valerate, benzoate, or dienanthate is effective in alleviating hot flushes (Campbell & Whitehead, 1977; Doring, 1976; Dusterberg & Nishino, 1982; Klopper, 1976; Lauritzen, 1973). Likewise, the synthetic alkylating estrogen, ethinyl E_2, is a potent and an effective contraceptive agent (Burkman, 1988). However, these estrogen compounds act upon all steroid-responsive tissues which limits their therapeutic efficacy. Moreover, since these steroids equilibrate among all body tissues, only a fraction of the administered dose accumulates at or near the site of action in the CNS. These properties of the estrogen then force either frequent dosing, or the administration of a depot form of the steroid, to maintain therapeutically effective levels in the brain. These treatment strategies lead to sustained increases in peripheral estrogen levels, and thus augment the risk of peripheral toxicities. The redox-based E_2-CDS may be potentially useful in the effective treatments of brain diseases by providing sustained and sufficient E_2 to the brain while avoiding peripheral toxicities.

The present studies were undertaken to determine if the E_2-CDS behaves as predicted on the basis of the physicochemical properties designed

into its structure. We determined (1) the effects of E_2-CDS dose on tissue concentrations of E_2-Q+ and E_2, (2) the effects of E_2-CDS dose on the rate of oxidation of E_2-CDS to E_2-Q+ and hydrolysis of E_2-Q+ to E_2, and (3) the effects of E_2-CDS dose on the clearance of E_2-Q+ and E_2 in a variety of tissues in the female rat. More specifically, our objective was to analyze quantitatively both E_2-Q+ and E_2 (two metabolites of the E_2-CDS) in brain, hypothalamus, anterior pituitary, kidney, lung, heart, liver, fat, uterus, and plasma following a single iv injection of one of several different doses of the E_2-CDS over a 28-day time course in ovariectomized (OVX) rats.

The rationale for using OVX female rat model in this study was twofold: 1) this animal model exhibits very low endogenous estrogen levels since both ovaries have been removed, thus allowing reliable determination of tissue metabolites of the E_2-CDS and 2) the results from this animal model would allow us to make comparison with the results obtained from the male rat model (Chapter 5) with regard to E_2-CDS distribution.

Materials and Methods

Experiment 1

To evaluate the dose- and time-dependent effects of E_2-CDS on the tissue distribution of E_2-Q+ and E_2 in female rats, all animals were bilaterally ovariectomized (OVX) under metofane anesthesia. All experiments were initiated exactly 2 weeks after ovariectomy. On day 15 after ovariectomy, rats (7 per group per each time point) were administered a single iv injection (tail vein) of the E_2-CDS at doses of 0 (HPCD), 0.01, 0.1, or 1.0 mg/kg body weight or E_2 at a dose of 0.7 mg/kg (equimolar to the 1.0-mg/kg dose of E_2-CDS).

Rats (7 per group) were killed by decapitation 1, 7, 14, 21, or 28 days after the drug administration and the trunk blood was collected in heparinized tubes. The blood was centrifuged and the plasma separated and stored at -20°C until hormone analysis. Tissues (whole brain, hypothalamus, anterior pituitary, kidney, lung, heart, liver, fat, and uterus) were dissected immediately following decapitation, rinsed in ice-cold saline, stripped of surrounding connective tissue where necessary, blotted dry on paper, and then stored at -80°C until hormone analysis.

Tissue samples of known wet weight at a concentration of 1 mg/20 µl solvent were processed and assayed for E_2-Q+ and E_2 by the method described in Chapter 4 (Rahimy et al., 1989a). Also, tissue homogenates or plasma from HPCD-treated rats were analyzed to determine residual E_2 concentrations and thereby served as our estimate of hormone background.

Experiment 2

To accurately assess the kinetics of E_2-CDS within the general circulation, an acute 7-day time-course study with frequent blood sampling was undertaken. To facilitate frequent blood sampling from unrestrained animals, a group of OVX rats were equipped with Silastic catheter (i.d. 0.5 mm, o.d. 1 mm). The catheter was positioned in the right atrium via the external jugular vein under pentobarbital anesthesia, according to the procedure described by Steffens (1969).

After recovery from the surgical procedure (usually one week, 3 weeks after OVX), rats were administered iv (via tail vein) with 1.0 mg/kg dose of the E_2-CDS. Immediately after drug treatment, animals were transferred to special sampling chambers for serial blood sampling. Blood samples (1 ml)

were removed at 0.5, 1, 2, 4, 8, 12, 24, 48, 96, and 168 hrs post-injection. At each sampling time, the blood was centrifuged and the plasma collected for E_2-Q^+ and E_2 analysis. Red cells were then resuspended in 0.5 ml heparinized saline (40 units/ml) and returned to each respective animal before the next blood sample. The volume of resuspended red cell solution was approximately 1 ml. It should be emphasized that although attempts were made to minimize the occurrence of potential problems associated with the design of this experiment, certain problems were unavoidable and thus should be mentioned. During the initial 12-hrs of the time course of this experiment, approximately 3 ml of plasma (i.e. 1/2 the total blood volume withdrawn) were collected from each animal. This volume of plasma was needed to analyze both metabolites of the E_2-CDS in plasma. However, this volume represents approximately 25% of total blood volume in an adult rat. And since red cells were resuspended in equal volume of heparinized saline and returned to each animal before the next blood sample, this procedure might decrease the plasma concentrations of the E_2-CDS metabolites.

Coat-A-Count Estradiol kits, a solid-phase [^{125}I]-radioimmunoassay, designed for the quantitative measurement of E_2 in serum were used for the assay of E_2 in all tissue samples. All the purified dried E_2 unknowns were reconstituted in 300 µl of the assay buffer (kit Zero Calibrator) and assayed in duplicate by the RIA. The intraassay and interassay coefficients of variation for E_2 were 1.56 and 6.1%, respectively. All samples were determined in 14 RIA runs.

Results

Experiment 1

To estimate the extent of *in vivo* oxidation of E_2-CDS to E_2-Q+, we determined for each tissue the magnitude of increase in E_2-Q+ concentrations over the 100-fold increase in E_2-CDS dose, at day 1 after injection (the first sampling time). The enzymatic oxidation of E_2-CDS to E_2-Q+ showed a clear dose dependency in brain, hypothalamus, plasma, kidney, lung, heart, liver, and fat tissues (Table 4). This dose-related oxidation ranged from 73-fold in liver to 176-fold in whole brain tissue over the 100-fold increase in E_2-CDS dose administered. The uterus was an exception and showed only a 21-fold increase in E_2-Q+ concentrations over the 100-fold increase in E_2-CDS dose (Table 4).

The *in vivo* rate of hydrolysis of E_2-Q+ to E_2 was estimated for each tissue at each dose of E_2-CDS administered by determining the ratio of E_2 to E_2-Q+ on the first sampling day (day 1, Table 4). This ratio remained constant over a 100-fold dose range for the hypothalamus, kidney, heart, and uterus; it decreased moderately (less than 50%) over the 100-fold dose range for the brain, lung, and liver and decreased precipitously for plasma and fat tissue (Table 4).

At 1 day after administration of E_2-CDS, all tissues showed a dose-dependent increase in concentrations of E_2-Q+ and E_2. Furthermore, the concentration-time profiles revealed a gradual decline in concentrations of E_2-Q+ and E_2 in whole brain (Figure 10) as well as in hypothalamus (Figure 11), with $t_{1/2}$ = 8-9 days. In contrast, both E_2-Q+ and E_2 were rapidly cleared

from plasma (Figure 12), liver (Figure 13), fat (Figure 14), and anterior pituitary, kidney, lung, heart, and uterus (Tables 5 and 6). By 28 days (the last sampling time) after a single injection of 1.0 mg E_2-CDS/kg, the E_2-Q+ concentrations remained elevated at 9.8 ± 0.7 ng/g wet tissue (mean ± SEM) in brain (Figure 10, left column, upper panel) and 10.6 ± 0.2 ng/g in hypothalamus (Figure 11, left column, upper panel). In contrast, peripheral tissues concentrations of E_2-Q+ were reduced to 2.9 ± 0.1 ng/g in anterior pituitary, 5.2 ± 2.2 ng/g in kidney, 2.9 ± 0.5 ng/g in lung, 1.7 ± 0.3 ng/g in heart, 2.5 ± 0.7 ng/g in uterus (Table 5, % reduction); and E_2-Q+ values were undetectable in plasma, liver, and fat (Figures 12-14, left columns, upper panels) 28 days after administration of 1.0 mg E_2-CDS/kg dose.

Similarly, E_2 concentrations were maintained relatively high in whole brain (Figure 10, right column of panels) and in hypothalamus (Figure 11, right column of panels); however, E_2 concentrations in peripheral tissues (except for anterior pituitary and plasma) fell by more than 80% by day 7, and by 97% by day 21, and were undetectable by day 28 (Table 6).

In contrast with E_2 concentrations achieved following E_2-CDS administration, E_2 levels in tissues following equimolar E_2 administration were remarkably low (Table 7) and in all tissues examined, the clearance of E_2 was rapid (Table 7). A comparison of the tissue levels of E_2 achieved at day 1 revealed that following E_2-CDS administration, brain (Figure 10) and hypothalamus (Figure 11) levels of E_2 were 88- and 22-fold greater, respectively, than levels observed in these tissues following 17 β-E_2 administration (Table 7). By 7 days after treatment, the E_2-CDS produced brain and hypothalamic E_2 concentrations that were 182- and 55-fold greater, respectively, than those achieved by an equimolar 17 β-E_2 dose.

Experiment 2

Figure 15 depicts the concentration-time profiles of the E_2-CDS metabolites (E_2-Q^+, upper panel; E_2, lower panel) in plasma of OVX rats. By 30 min after administration of the E_2-CDS, plasma E_2-Q^+ increased to (61.9 ± 3.8 ng/ml). The E_2-Q^+ levels decreased by 50% at 8 hrs and by greater than 88% at 24 hrs after E_2-CDS treatment. Kinetic analysis revealed that the plasma E_2-Q^+ concentration-time profile fits a sum of two exponentials. The half-lives of these two phases were $t_{1/2}$ = 8.16 and $t_{1/2}$ = 70.38 hrs respectively in plasma. The area under curve (AUC_t) was 835.25 ng/ml hr for the time-course studied.

Similarly, plasma E_2 concentrations were increased to 1.9 ± 0.08 ng/ml after 30 min of E_2-CDS administration. The plasma E_2 levels decreased by 50% at 3 hrs and by greater than 91% at 24 hrs after E_2-CDS treatment. The plasma E_2 concentration-time profile was best fitted in a sum of three exponentials. The half-lives of the three phases were $t_{1/2}$ = 0.14, $t_{1/2}$ = 2.35, $t_{1/2}$ = 38.99 hrs, respectively. The area under curve (AUC_t) was 23.85 ng/ml hr for the time-course studied. The third half-life indicated the presence of deep compartment (most likely the brain) which slowly releases E_2 in plasma.

Discussion

This detailed dose-distribution and time-course study demonstrates that (i) the enzymatic oxidation of E_2-CDS to E_2-Q^+ is dose dependent, and with the possible exception of the uterus, the oxidation is not saturable over the 100-fold dose range tested; (ii) the hydrolysis of E_2-Q^+ to E_2 was dependent upon the tissues analyzed and appeared to be saturable only in plasma and fat

and, to a lesser extent, in brain, lung, and liver; and (iii) the disappearance of both E_2-Q+ and E_2 was slow in brain tissue and rapid in all peripheral tissues tested. Collectively, these data are consistent with the expected behavior of the E_2-CDS (Bodor et al., 1987).

Dose and time-course profiles revealed that E_2-Q+ persists in brain tissue as well as in hypothalamus, with a $t_{1/2}$ = 8-9 days, but it is rapidly cleared from the periphery. The previous single-dose distribution study in intact male rats (Chapter 5) demonstrated a similar half-life for E_2-Q+ in brain tissue. These estimates of the half-life of E_2-Q+ in the brain are in accordance with reports which utilized different analytical techniques and E_2-CDS doses (Mullersman et al., 1988). The slow clearance of the E_2-CDS metabolite, E_2-Q+, from the CNS tissue appears to be independent of dose since similar values have been obtained in studies using doses of E_2-CDS ranging from 0.01 mg/kg (present report) to 15 mg/kg dose (Mullersman et al., 1988). Further, the long half-life of E_2-Q+ in brain tissue does not appear to be an artifact of its sustained production from E_2-CDS since the half-life of the delivery system itself in brain tissue is only 29.2 min, indicating rapid oxidation to E_2-Q+ (Bodor et al., 1987). Thus, as predicted based on the physicochemical properties of the E_2-CDS (Bodor et al., 1987), the unique features of the BBB appear to contribute to the chronic retention by the brain of the charged, hydrophilic E_2-Q+.

The anterior pituitary exhibited slower elimination of the metabolites of E_2-CDS (E_2-Q+ & E_2) than other peripheral tissues. By 7 days following administration of a 1.0-mg E_2-CDS dose, the hypothalamic-anterior pituitary E_2-Q+ ratio was 1.3 and then increased to about 4-fold by 28 days. Similarly, the hypothalamic-anterior pituitary E_2 ratio was 1.2 on day 7 and this ratio was maintained throughout the 28-day time course. This relative persistency

of both E_2-Q+ and E_2 in the anterior pituitary may be caused by the anatomical relationship between the hypothalamus and the pituitary gland. Estradiol released upon the hydrolysis of E_2-Q+, or the E_2-Q+ itself, which is locked into brain, could be delivered directly to the pituitary by the capillary plexus of the hypophyseal portal system. These capillaries in the median eminence lack features of other brain capillaries and hence are not part of the BBB (Traystman, 1983). Thus, the median eminence would not be expected to prevent the efflux of E_2-Q+ from brain, and transfer of E_2-Q+ to anterior pituitary can be expected.

Plasma also showed, after day 1, a residual but detectable E_2 concentration throughout the time course of the 1.0-mg E_2-CDS dose and through the 21-day time course of the 0.1-mg dose. This prolonged and residual E_2 in plasma is likely to be the result of a continuous redistribution of E_2 liberated from E_2-Q+ in the brain or other tissues down its concentration gradient into the general circulation.

The oxidation of E_2-CDS to E_2-Q+ in uterine tissue did not appear to be dose dependent, since a 100-fold increase in dose resulted in a 21-fold increase in E_2-Q+ concentration. This observation is perhaps, in part, an artifact of the observed uterine hypertrophy in animals treated with the E_2-CDS (Anderson et al., 1988a). At day 1 after E_2-CDS treatment, a 100-fold increase in E_2-CDS dose resulted in 54% increase in uterine weight (Chapter 7, Table 8), this might reduce the values for E_2-Q+ when normalized for tissue weight. However, when the extent of oxidation was estimated per tissue, we observed only a 32-fold increase over the 100-fold dose range examined. Additionally, since blood flow to the uterus of OVX rats is low, the expected rapid oxidation of E_2-CDS to E_2-Q+ would occur less likely in that tissue. Finally, we cannot rule out the possibility that in the uterus of OVX rats, the enzymatic oxidation

of E_2-CDS to E_2-Q+ is a saturable process and is thereby independent of the dose of E_2-CDS administered.

To demonstrate further the preferential deposition and retention of estrogen in the CNS with the E_2-CDS, one dose of 17 β-E_2 (equimolar to the 1.0-mg E_2-CDS dose) was also studied. As shown in Table 7, E_2 concentrations in the CNS tissues of rats treated with 17 β-E_2 were slightly increased on day 1, and were just above the detection limits of the assay at 7 days. In contrast, the 1.0 mg E_2-CDS dose resulted in brain E_2 concentrations that were 81- and 182-fold greater than those achieved following 17 β-E_2 injection at 1 and 7 days, respectively. These data demonstrated that the E_2-CDS is much more effective than 17 β-E_2 itself in delivering and retaining the estrogen in the brain.

Collectively, these observations are consistent with the proposal that E_2 can be preferentially delivered to the brain using a redox-based chemical delivery system, an inert molecule which requires several steps in its conversion to the parent drug (Bodor et al., 1981; Bodor & Brewster, 1983). The multiple, facile enzymatic conversions including oxidation and hydrolytic cleavage may not only lead to preferential E_2 delivery and sustained release/effects, but may also act to decrease the toxicity of the drug. A preferential and sustained CNS estrogen delivery can be potentially useful since estrogens are known to influence a variety of CNS functions (McEwen, 1988; Maggi & Perez, 1985).

Figure 10. Dose and time-dependent effects of the E_2-CDS on E_2-Q+ concentrations (left column of panels) and E_2 concentrations (right column of panels) in whole brain of ovariectomized rats. Animals received a single iv (tail vein) injection of the E_2-CDS on day 0 at a dose of 1.0 mg/kg (upper panels), 0.1 mg/kg (middle panels), or 0.01 mg/kg (lower panels). Animals were killed 1, 7, 14, 21, or 28 days after the drug administration. Tissue samples of known wet weight at a concentration of 1 mg/20 µl solvent were processed and assayed for E_2-Q+ and E_2 by the method described in Chapter 4. Also, tissue homogenates from HPCD-treated rats were analyzed for E_2 hormone background. Represented are means ± SEM for n = 7 rats per group per sampling time.

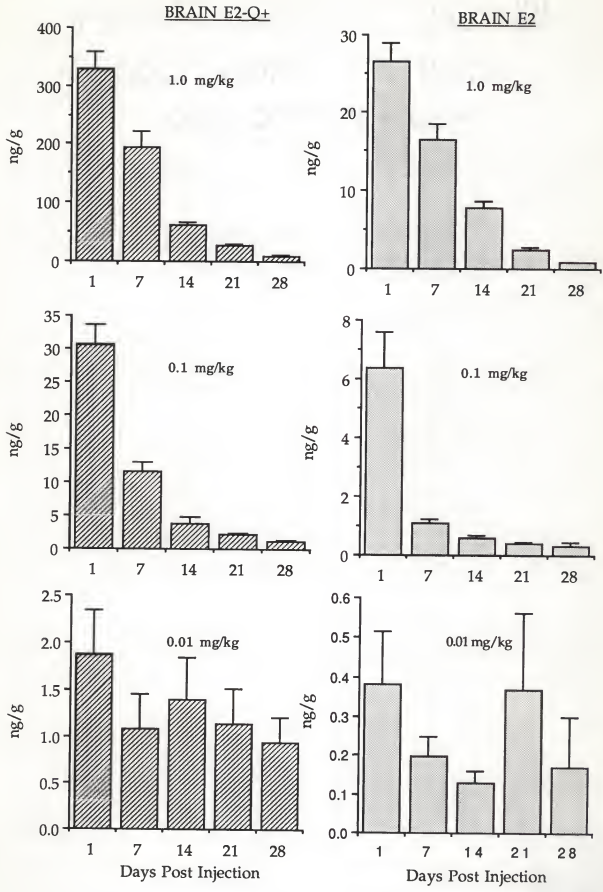

BRAIN E2-Q+
BRAIN E2
1.0 mg/kg
0.1 mg/kg
0.01 mg/kg
ng/g
Days Post Injection

Figure 11. Dose and time-dependent effects of the E_2-CDS on E_2-Q^+ concentrations (left column of panels) and E_2 concentrations (right column of panels) in hypothalamus of ovariectomized rats. Animals received a single iv (tail vein) injection of the E_2-CDS on day 0 at a dose of 1.0 mg/kg (upper panels), 0.1 mg/kg (middle panels), or 0.01 mg/kg (lower panels). Animals were killed 1, 7, 14, 21, or 28 days after the drug administration. Tissue samples of known wet weight at a concentration of 1 mg/20 µl solvent were processed and assayed for E_2-Q^+ and E_2 by the method described in Chapter 4. Also, tissue homogenates from HPCD-treated rats were analyzed for E_2 hormone background. Represented are means ± SEM for n = 7 rats per group per sampling time.

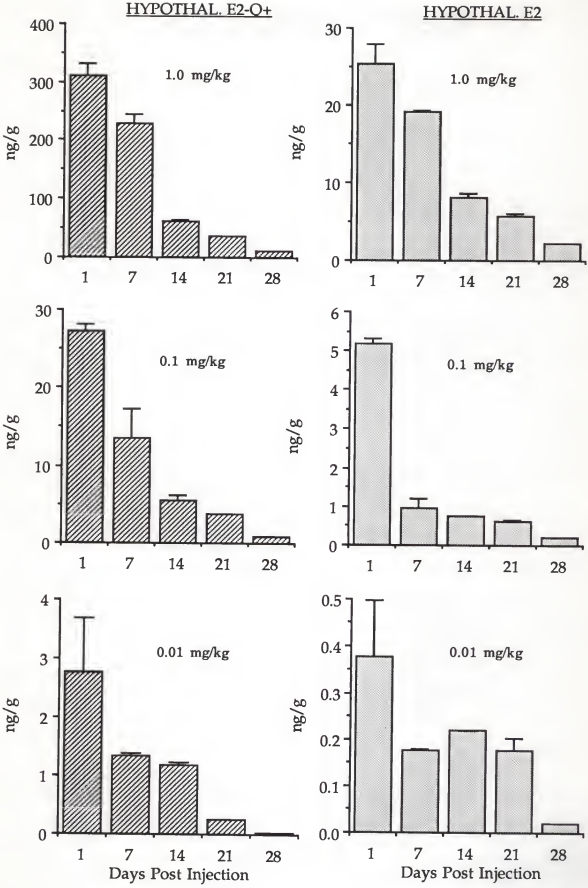

HYPOTHAL. E2-Q+
HYPOTHAL. E2
1.0 mg/kg
1.0 mg/kg
0.1 mg/kg
0.1 mg/kg
0.01 mg/kg
0.01 mg/kg
ng/g
400
300
200
100
0
30
20
10
6
5
4
3
2
1
0.5
0.4
0.3
0.2
0.1
0.0
1
7
14
21
28
Days Post Injection

Figure 12. Dose and time-dependent effects of the E_2-CDS on E_2-Q^+ concentrations (left column of panels) and E_2 concentrations (right column of panels) in plasma of ovariectomized rats. Animals received a single iv (tail vein) injection of the E_2-CDS on day 0 at a dose of 1.0 mg/kg (upper panels), 0.1 mg/kg (middle panels), or 0.01 mg/kg (lower panels). Animals were killed 1, 7, 14, 21, or 28 days after the drug administration. Plasma samples of known aliquot were processed and assayed for E_2-Q^+ and E_2 by the method described in Chapter 4. Also, plasma samples from HPCD-treated rats were analyzed for E_2 hormone background. Represented are means ± SEM for n = 7 rats per group per sampling time. * Indicates below the sensitivity limit of the assay.

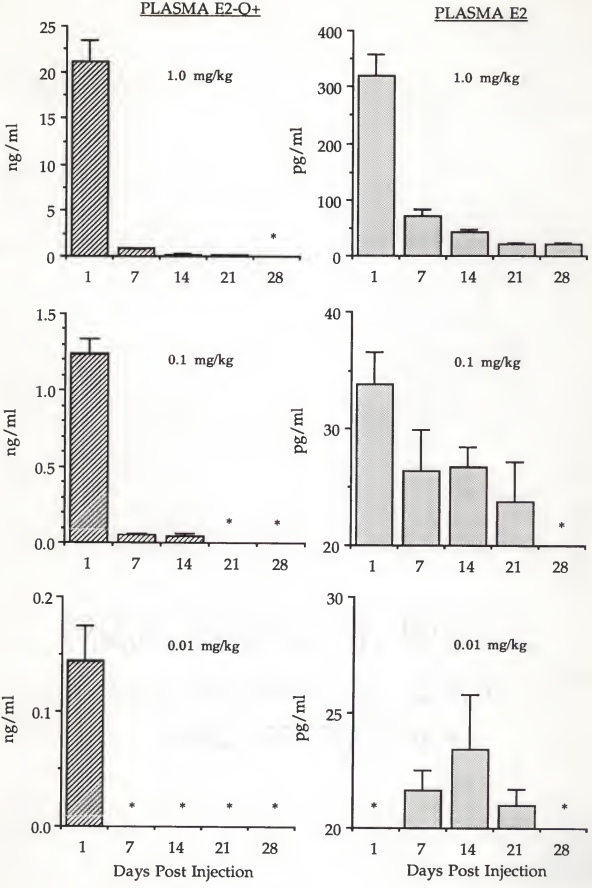

PLASMA E2-Q+
PLASMA E2
1.0 mg/kg
0.1 mg/kg
0.01 mg/kg
ng/ml
pg/ml
Days Post Injection
Days Post Injection

Figure 13. Dose and time-dependent effects of the E_2-CDS on E_2-Q+ concentrations (left column of panels) and E_2 concentrations (right column of panels) in liver of ovariectomized rats. Animals received a single iv (tail vein) injection of the E_2-CDS on day 0 at a dose of 1.0 mg/kg (upper panels), 0.1 mg/kg (middle panels), or 0.01 mg/kg (lower panels). Animals were killed 1, 7, 14, 21, or 28 days after the drug administration. Tissue samples of known wet weight at a concentration of 1 mg/20 µl solvent were processed and assayed for E_2-Q+ and E_2 by the method described in Chapter 4. Also, tissue homogenates from HPCD-treated rats were analyzed for E_2 hormone background. Represented are means ± SEM for n = 7 rats per group per sampling time. * Indicates below the sensitivity limit of the assay.

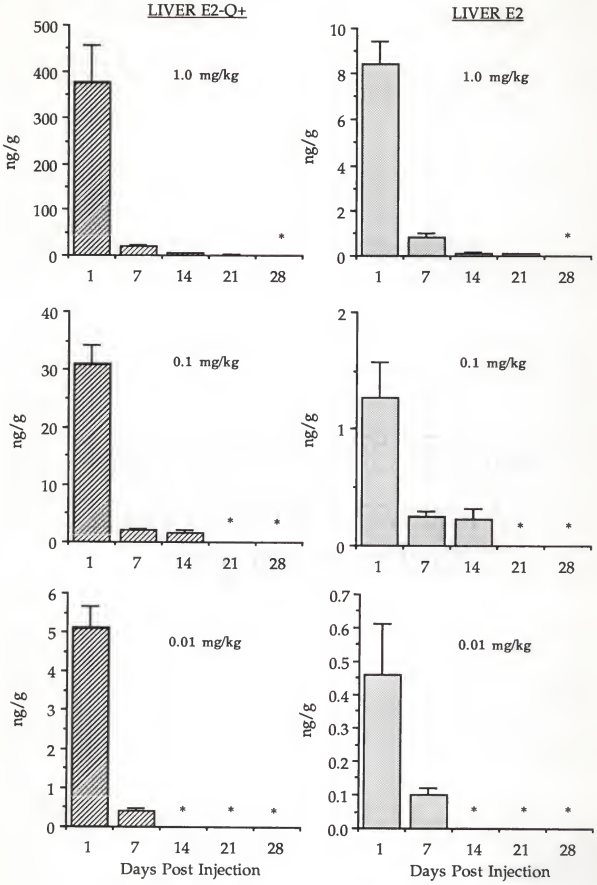
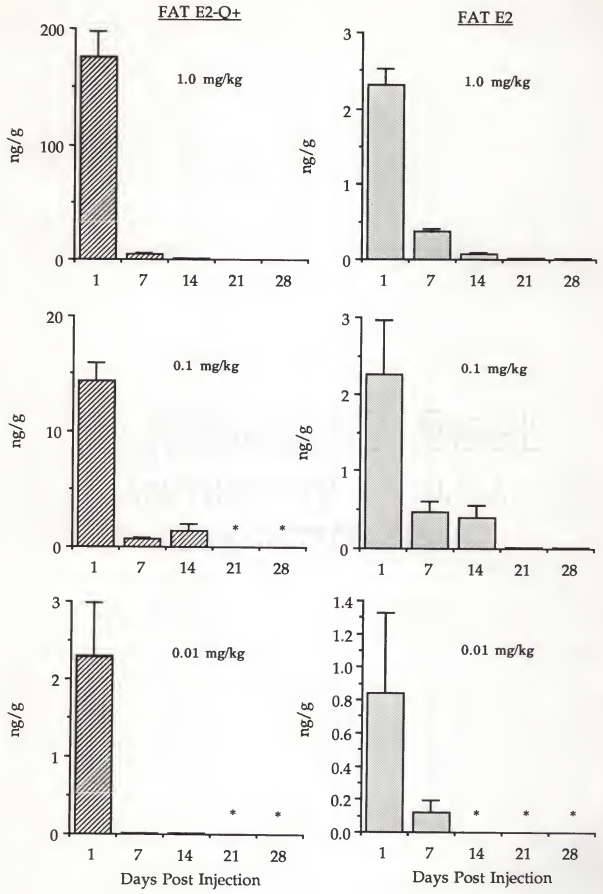

LIVER E2-Q+
LIVER E2
1.0 mg/kg
0.1 mg/kg
0.01 mg/kg
ng/g
Days Post Injection
1
7
14
21
28
*

Figure 14. Dose and time-dependent effects of the E_2-CDS on E_2-Q^+ concentrations (left column of panels) and E_2 concentrations (right column of panels) in fat of ovariectomized rats. Animals received a single iv (tail vein) injection of the E_2-CDS on day 0 at a dose of 1.0 mg/kg (upper panels), 0.1 mg/kg (middle panels), or 0.01 mg/kg (lower panels). Animals were killed 1, 7, 14, 21, or 28 days after the drug administration. Tissue samples of known wet weight at a concentration of 1 mg/20 µl solvent were processed and assayed for E_2-Q^+ and E_2 by the method described in Chapter 4. Also, tissue homogenates from HPCD-treated rats were analyzed for E_2 hormone background. Represented are means ± SEM for n = 7 rats per group per sampling time. * Indicates below the sensitivity limit of the assay.


FAT E2-Q+
FAT E2
1.0 mg/kg
0.1 mg/kg
0.01 mg/kg
ng/g
Days Post Injection
1
7
14
21
28
*

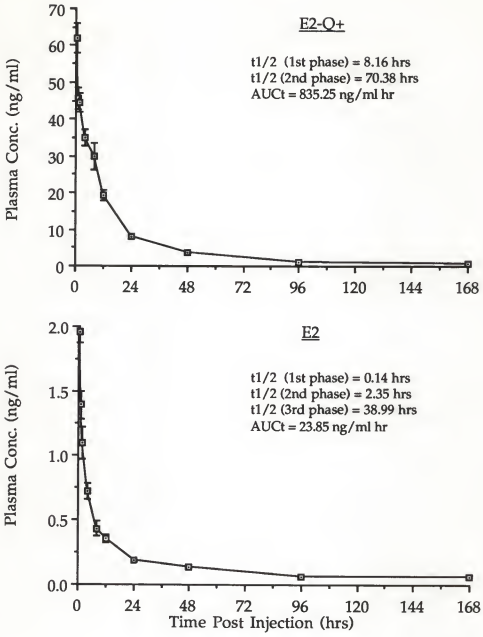


Figure 15. Effects of a single iv dose of the E_2-CDS (1.0 mg/kg bw) on plasma E_2-Q+ (upper panel) or plasma E_2 (lower panel) concentrations in ovariectomized rats. Animals received a single iv (tail vein) injection of the E_2-CDS on day 0 at a dose of 1.0 mg/kg. Immediately after drug treatment, animals were transferred to sampling chambers and blood samples were removed at 0.5, 1, 2, 4, 8, 12, 24, 48, 96, and 168 hrs postinjection. Plasma samples were assayed for E_2-Q+ and E_2 as described in Chapter 4. Represented are means ± SEM for n = 5 rats per group per sampling time.

Table 4: Effects of Dose on the Extent of Oxidation and Hydrolysis of E_2-CDS in a Variety of Tissues *in vivo*

Tissue	Oxidation (-fold increase)[a]	Hydrolysis[b] E_2-CDS Dose (mg/kg)		
		0.01	0.1	1.0
Brain	176	24	23	13
Hypothalamus	112	22	23	19
Ante. Pituitary	ND[c]	40	31	ND
Plasma	147	14	4	2
Kidney	103	29	22	27
Lung	116	47	32	24
Heart	125	25	21	29
Liver	73	13	15	6
Fat	77	35	22	7
Uterus	21	31	32	26

a Fold increase in E_2-Q+ concentrations over a 100-fold increase in the E_2-CDS dose at day 1 after injection (the first sampling time).

b Average % hydrolysis (fraction of E_2-Q+ hydrolyzed ÷ total E_2-Q+ x 100) on the first sampling time (day 1).

c Not determined due to incorrect extraction of the tissue at day 1.

Table 5: Effects of the E_2-CDS on the Clearance of E_2-Q+ from a Variety of Tissues

Tissue	Dose (mg/kg)	Days after treatment				
		1[a]	7[b]	14[b]	21[b]	28[b]
Brain	1.0	329.97	42	82	92	97
Hypothalamus	1.0	310.58	16	80	88	96
Anterior Pituitary	1.0	ND[c]	ND	ND	ND	ND
Plasma	1.0	21.11	96	99	>99	UD[d]
Kidney	1.0	487.56	80	96	98	>99
Lung	1.0	716.94	83	98	99	>99
Heart	1.0	910.39	82	98	>99	>99
Liver	1.0	374.57	94	98	>99	UD
Fat	1.0	176.11	97	99	>99	UD
Uterus	1.0	92.58	89	93	97	>98

a Initial concentrations of E_2-Q+ (ng/g wet tissue or ng/ml) 1 day after administration of the E_2-CDS.

b % reduction in E_2-Q+ concentrations at various times after E_2-CDS treatment relative to the initial corresponding values.

c Not determined due to incorrect extraction of the tissue at day 1.

d Undetectable (below the sensitivity of RIA for E_2).

Table 6: Effects of the E_2-CDS on the Clearance of E_2 from a Variety of Tissues

Tissue	Dose (mg/kg)	Days after treatment				
		1[a]	7[b]	14[b]	21[b]	28[b]
Brain	1.0	26.63	40	71	91	96
Hypothalamus	1.0	25.32	24	68	77	91
Anterior Pituitary	1.0	ND[c]	ND	ND	ND	ND
Plasma	1.0	0.32	78	87	93	95
Kidney	1.0	135.17	80	98	>99	UD[d]
Lung	1.0	167.67	95	99	>99	>99
Heart	1.0	156.03	82	99	>99	>99
Liver	1.0	8.43	90	98	>99	UD
Fat	1.0	2.32	84	97	>99	>99
Uterus	1.0	23.07	93	94	97	>99

a Initial concentrations of E_2 (ng/g wet tissue or ng/ml) 1 day after administration of the E_2-CDS.

b % reduction in E_2 concentrations at various times after E_2-CDS treatment relative to the initial corresponding values.

c Not determined due to incorrect extraction of the tissue at day 1.

d Undetectable (below the sensitivity of RIA for E_2).

Table 7: Effects of an Equimolar Dose of E_2 (0.7 mg/kg) on the Tissue Concentrations of E_2[a]

Tissue	Vehicle (HPCD)[b]	Days after treatment	
		1[c]	7[c]
Brain	0.05 ± 0.03	0.33 ± 0.04	0.09 ± 0.07
Hypothalamus	0.03 ± 0.01	1.16 ± 0.14	0.35 ± 0.08
Ante. Pituitary	0.02 ± 0.01	0.99 ± 0.07	0.28 ± 0.02
Plasma	UD[d]	0.03 ± 0.01	0.02 ± 0.01
Kidney	UD	0.55 ± 0.13	0.04 ± 0.01
Lung	UD	0.26 ± 0.30	0.01 ± 0.01
Heart	UD	0.14 ± 0.04	0.01 ± 0.01
Liver	0.02 ± 0.01	0.80 ± 0.20	0.11 ± 0.04
Fat	0.05 ± 0.03	1.12 ± 0.29	0.11 ± 0.05
Uterus	UD	2.08 ± 0.65	0.17 ± 0.14

a This dose of E_2 (0.7 mg/kg) is equimolar to 1.0 mg E_2-CDS/kg dose.
b Residual E_2 concentrations (ng/g wet tissue or ng/ml; mean ± SEM).
c E_2 concentrations (ng/g wet tissue or ng/ml; mean ± SEM) at various times following administration of E_2.
d Undetectable (below the sensitivity of RIA for E_2).
Note: For the determination of E_2 concentration in this table, we used 200 to 300 mg tissue to obtain a detectable E_2 baseline in the RIA since tissue E_2 concentration was very low following 17 β-E_2 injection. And since our assay methodology was optimized for up to 100 mg tissue, using excess amount of tissue in the assay might result in unreliable E_2 values following 17 β-E_2 administration.

CHAPTER 7
EVALUATION OF THE PHARMACODYNAMIC EFFECTS OF E_2-CDS IN OVARIECTOMIZED FEMALE RATS

Introduction

Estrogen hormones have been shown to influence a myriad of CNS processes including reproductive parameters, i.e., neuroendocrine modulation of reproductive cycle (Kalra & Kalra, 1989; Maggi & Perez, 1985; Plant, 1986) and stimulation of sexual behaviors (Beyer et al., 1976; Christensen & Clemens, 1974) as well as non-reproductive parameters such as regulation of neurotransmissions involved in sensorimotor functions, mood, and learning tasks (Fillit et al., 1986; McEwen et al., 1984; Smith, 1989). These diverse actions of estrogens on the CNS functions are of significant therapeutic interest after menopause or ovariectomy when endogenous estrogens decline. In these and certain other cases, since estrogen medications are primarily used for their central actions, the preferential brain estrogen delivery is not only beneficial but may produce safer and more potent natural therapeutic agent.

The evaluation of the tissue distribution patterns of both E_2-Q^+ and E_2 in intact male (Chapter 5; Rahimy et al., 1990a) as well as in OVX female rats (Chapter 6; Rahimy et al., 1990b) substantiated the major aspect of the proposed mechanism of this redox-based estrogen delivery system for the brain. That is, the E_2-CDS consistently demonstrated its predictive pharmacokinetic behaviors including the preferential retention of E_2-Q^+ and thus, E_2 in the CNS tissue with $t_{1/2}$ = 8-9 days, while simultaneously

accelerated the elimination of these metabolites from the peripheral tissues. On the basis of these pharmacokinetic findings, the E_2-CDS is expected to exhibit pharmacodynamic responses with long duration of effects following a single administration of the delivery system.

The present study was undertaken to determine whether the long half-lives and the magnitude of E_2-CDS metabolites in brain tissue (Chapters 5 & 6) correlate with the duration of pharmacodynamic effects. More specifically, the objectives were (1) to assess the dose- and time-dependent effects of the E_2-CDS on brain-mediated responses, i.e. anterior pituitary hormones secretion in OVX rats; (2) to compare E_2-CDS with an equimolar dose of 17 β-E_2; and (3) to correlate the half-lives of the E_2-CDS metabolites with the duration of pharmacodynamic effects mediated by E_2.

Materials and Methods

All the samples analyzed in this study were obtained from the animals used and described in the preceding study (Chapter 6); this chapter presents further data on evaluation of the pharmacodynamic responses of E_2-CDS. Briefly, rats were ovariectomized (OVX) and two weeks later were administered a single iv injection of the E_2-CDS at doses of 0 (HPCD), 0.01, 0.1, or 1.0 mg/kg body weight or E_2 at a dose of 0.7 mg/kg (equimolar to the 1.0 mg E_2-CDS dose). Animals (7 per group) were then killed by decapitation 1, 7, 14, 21, or 28 days after the drug administration and plasma, anterior pituitary, and uterine tissues were collected for subsequent analysis.

Plasma luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH), and prolactin (PRL) concentrations were measured in duplicate by the RIA using NIDDK kits. Plasma LH, FSH and GH values are

expressed as ng/ml of either the LH-RP-2, FSH-RP-2, or GH-RP-2 reference standard, respectively and PRL values are expressed as ng/ml of the PRL-RP-3 standard. The intra-assay coefficients of variation were 4.67%, 5.02%, 4.05%, and 4.96% for LH, FSH, GH, and PRL assays, respectively. All the samples for each hormone were assayed in a single run.

Results

The E_2-CDS caused a dose- and time-dependent suppression of plasma LH throughout the time-course studied (Figure 16). The maximum LH reduction occurred at 7 days postinjection. At this time, LH was suppressed by 21, 46 and 86% relative to HPCD control at doses of 0.01, 0.1 and 1.0 mg E_2-CDS/kg, respectively (Figure 16). The plasma LH concentrations in animals treated with 1.0 mg E_2-CDS were significantly reduced by 56, 86, 72, and 56% at 1, 7, 14, or 21 days, respectively and remained suppressed by greater than 35% at 28 days after drug administration. By contrast, equimolar E_2 dose (0.7 mg/kg) caused a transient reduction in LH concentrations of 27% on day 1 and 24% on day 7 which were not significantly different from time 0 values (Figure 16).

Similarly, the E_2-CDS caused a dose- and time-dependent suppression of plasma FSH throughout the time-course studied (Figure 17). The maximum FSH reduction occurred at 7 days postinjection. FSH was suppressed by 14, 28, and 58% relative to control at doses of 0.01, 0.1 and 1.0 mg E_2-CDS/kg, respectively, on day 7 (Figure 17). The plasma FSH concentrations in animals treated with 1.0 mg E_2-CDS were significantly reduced by 37, 58 and 20% at 1, 7 or 14 days, respectively and by 7% (day 21) or were at preinjection levels by 28 days after drug administration. By contrast,

equimolar E_2 dose reduced plasma FSH by 27% at day 1 and 19% at day 7 (Figure 17).

Plasma concentrations of LH and FSH in animals treated with lower doses of the E_2-CDS (0.01 and 0.1 mg/kg) began to gradually increase after 7 days of drug administration (Figures 16 &17).

Plasma PRL concentrations in animals treated with 1.0 mg E_2-CDS dose were increased by 4-, 8-, 13-, and 8-fold at 1, 7, 14 and 21 days, respectively or were at preinjection levels by 28 days after drug administration (Figure 18, upper panel). Lower doses of the E_2-CDS did not effect PRL concentrations. By contrast, the 0.7 mg/kg dose of E_2 increased plasma PRL concentrations by 3-fold on day 1 and PRL returned to preinjection levels by day 7 after drug administration (Figure 18, upper panel).

Plasma GH concentrations were not altered in response to E_2 or E_2-CDS at any dose or time point evaluated (Figure 18, lower panel).

Anterior pituitary weights increased in a dose- and time-dependent manner in response to E_2-CDS administration (Table 8). With the lower doses of the E_2-CDS (0.01 and 0.1 mg/kg), pituitary weights were slightly increased (22 to 32%) over control group weights by 14 days postinjection, but they returned to control levels by day 21. However, the highest dose of the E_2-CDS increased pituitary weights significantly from day 7 to day 28 relative to weights at time 0 and following treatment with lower doses of E_2-CDS. The maximum pituitary gland stimulation occurred at 14 days postinjection and then pituitary weights began to decrease but remained elevated at 28 days after the drug administration (Table 8).

Similarly, uterine weights showed a dose- and time-dependent increase in response to E_2-CDS administration (Table 8). Uterine weights were increased by 20, 54, or 82% on day 1 following treatment with the E_2-CDS at

0.01, 0.1, and 1.0 mg/kg doses, respectively. With the highest dose of the E_2-CDS (1.0 mg/kg), uterine weights were significantly increased by about 3-fold on day 7 which then weights began to decrease but remained elevated at 28 days after the drug administration (Table 8). It should be noted that even at the highest dose (1.0 mg E_2-CDS/kg), uterine weights were less than those typically observed in gonad-intact rats (500-625 mg).

An equivalent increase in anterior pituitary as well as uterine weights was observed with E_2 (0.7 mg/kg) compared to 1.0 mg E_2-CDS dose on day 1. However, by day 7 the effects of equimolar E_2 were equivalent to the lowest dose of E_2-CDS (0.01 mg/kg).

Discussion

The results of this study demonstrated that the E_2-CDS causes a dose- and time-dependent suppression of gonadotropin (LH & FSH) secretion in OVX rats with maximum reductions in plasma LH and FSH concentrations occurring 7 days after E_2-CDS administration. The time course of gonadotropin suppression in OVX rats is comparable to that previously observed for E_2-CDS effects on other parameters and in other animal models. We have reported long-term suppression of LH in castrated male rats (Estes et al., 1987b; Simpkins et al., 1986), in OVX female rats (Anderson et al., 1988a), and stimulation of masculine sexual behavior in castrated male rats for 28 days (Anderson et al., 1987a), and body weight alterations for 36 days (Simpkins et al., 1988) following a single iv administration of the E_2-CDS. Sarkar et al. (1989) have reported on the suspension of estrous cycles in female rats for 30 days following E_2-CDS treatment. These prolonged pharmacological effects further support the idea that the intermediate

metabolite of the E_2-CDS, E_2-Q+, is "locked" behind the BBB and there it serves as a brain depot for E_2 (Bodor, et al., 1987). From this store of E_2-Q+, E_2 is then slowly released through non-specific hydrolysis of the carrier, resulting in sustained brain exposure to E_2.

Since 17-substituted estrogens, such as the E_2-CDS and E_2-Q+, do not effectively bind to E_2 receptors (Dusterberg & Nishino, 1982; Janoko et al., 1984), they are not likely to exhibit estrogenic activity. Thus, it is reasonable to believe that neither the E_2-CDS nor the E_2-Q+ formed in the brain account for the prolonged pharmacological effects of this delivery system. Rather locally released E_2 in the brain, particularly the hypothalamus, would appear to account for the sustained suppression of the gonadotropin secretion.

Our previous evaluation of tissue distribution of the E_2-CDS in male rats (Chapter 5; Rahimy et al., 1990a) and the more detailed dose-response and time-course evaluation of the E_2-CDS distribution in OVX rats (Chapter 6; Rahimy et al., 1990b) revealed that (i) E_2-Q+, the quaternary form of E_2-CDS, as well as E_2 persists in the brain with $t_{1/2}$ = 8-9 days and (ii) the same metabolites are rapidly eliminated from the peripheral tissues. These findings together with the absence of a physiologically significant elevation of plasma E_2 concentrations from 7-28 days after the E_2-CDS administration (Chapter 6), provide strong evidence for the local action of E_2 in the brain, presumably on hypothalamic luteinizing hormone-releasing hormone (LHRH) containing neurons (Sarkar et al., 1989).

The synthesis and secretion of gonadotropins from the anterior pituitary are differentially regulated by several neuronal (Barraclough & Wise, 1982; Dalkin et al., 1989; Marshal & Kelch, 1986; Plant, 1986) and hormonal (Kalra & Kalra, 1980, 1983) factors including the hypothalamic decapeptide, LHRH, and the action of E_2 both in a positive and negative

feedback mode at the hypothalamus as well as the anterior pituitary. The evaluation of the effects of E_2-CDS on LHRH neuronal activity (i.e. LHRH release) showed that portal blood concentrations of LHRH were significantly reduced for more than 16 days following the treatment (Sarkar et al., 1989). The reduced LHRH secretion, was in contrast to the increased hypothalamic LHRH concentrations, suggesting that the inhibition of release resulted in a tissue buildup of the decapeptide. Furthermore, chronic exposure to E_2 has no significant effects on anterior pituitary responsiveness to LHRH (Cooper et al., 1974), indicating that the prolonged inhibitory effects of E_2-CDS on LH and FSH are due primarily to sustained suppression of LHRH secretion from the hypothalamus.

When the dynamics of the E_2-CDS effects were compared with that of an equimolar dose of E_2, the E_2-CDS showed 100-fold greater efficacy in the magnitude of inhibition of plasma LH and FSH. In other words, the magnitude of E_2 effects was equivalent to that of the E_2-CDS but with 100-fold lower dose (Figure 15). This marked increased in effectiveness and the prolonged duration of the E_2-CDS effects on LH and FSH secretion are most likely due to "lock-in" of the E_2-Q+ behind the BBB with subsequent slow release of E_2 in the brain.

When the kinetic behaviors of E_2-CDS and E_2 were compared on molar basis, the E_2-CDS (1.0 mg/kg) produced E_2 concentrations in brain tissue which were 81- and 182-fold greater than after an equimolar E_2 (0.7 mg/kg) treatment at 1 and 7 days postinjection, respectively (Chapter 6). Therefore, it seems more reasonable to suggest that following the E_2-CDS administration, the brain E_2 is continuously produced and as such the steady-state brain concentrations of E_2 is dependent on its rate of production from the E_2-Q+ and

its rate of elimination from the brain by either local metabolism or its redistribution down a concentration gradient into the general circulation.

We observed a significant elevation in plasma PRL in response to the highest dose of E_2-CDS (1.0 mg/kg), whereas lower doses had no significant effect on plasma PRL concentrations. It appears then that elevations in plasma PRL correlate with the administration of E_2-CDS at doses which result in the transient elevation of plasma E_2 levels, but not at doses at which plasma E_2 remains low (Chapter 6). This apparent stimulation of PRL production by the E_2-CDS would appear to be due to the well described actions of E_2 on the anterior pituitary lactotrophes (Chen & Meites, 1970). However, the lack of a positive temporal correlation between plasma PRL (present study) and plasma E_2 levels (Chapter 6) suggests the possibility that E_2 released in the brain might be responsible for a direct stimulation of the anterior pituitary. This can be explained by the anatomical relationship between the hypothalamus and the anterior pituitary gland. Estradiol released from the E_2-Q^+, or the E_2-Q^+ itself, which is "locked" into the brain, could be delivered directly to the anterior pituitary by the capillary plexus of the hypophyseal portal system (Traystman, 1983). These capillaries in the median eminence lack features of other brain capillaries and hence are not part of the BBB (Traystman, 1983).

The E_2-CDS had no significant effects on the mean plasma GH concentrations over the 28 days time-course at any of the 3 doses examined. However, a careful analysis of the effects of E_2-CDS on pulsatile GH secretion (Millard et al., 1990) revealed that while mean GH levels are not changed, baseline GH values were elevated and GH pulse amplitudes were moderately reduced at 7 days after E_2-CDS administration.

The marked increases in anterior pituitary weights of OVX rats treated with the E_2-CDS appears to be due to the direct effects of E_2 on the pituitary gland. These effects of E_2 appear to be exerted on the lactotroph population of the anterior pituitary (Chen & Meites, 1970; Gorski, 1981). E_2 is well known to stimulate PRL secretion and to induce hyperplasia of lactotrophs (Chen & Meites, 1970; Gorski, 1981). As indicated above, brain E_2 likely reaches the anterior pituitary gland, through the redistribution of the steroid down the marked concentration gradient from the brain to the pituitary gland (Traystman, 1983). It should be noted, however, that the effects of E_2-CDS on pituitary weight are dependent upon the OVX condition of the rats. In gonad-intact rats, E_2-CDS does not alter anterior pituitary weight.

The uterotrophic effects of E_2-CDS were also dose- and time-dependent. This effect of E_2-CDS likely relates to the extreme sensitivity of OVX rats to circulating estrogens (Mayer et al., 1960). Thus, even modest elevations in plasma E_2 following administration of E_2 or E_2-CDS (Chapter 6), result in stimulation of uterine tissue in OVX rats. However, both the uterus and the anterior pituitary gland of gonad-intact rats are unresponsive to the estrogen delivery system (Anderson et al., 1988a). Finally, it should be noted that the uterine weights observed following E_2-CDS were considerably lower than the 500 to 625 mg weights normally seen in gonad-intact rats (Anderson et al., 1988a).

In conclusion, the prolonged effects of the E_2-CDS on gonadotropins suppression were dose- and time-dependent, and the duration of these responses are consistent with the long half-lives of the E_2-CDS metabolites in the brain. These results further support the view that the E_2-CDS may be potentially useful in fertility regulation and treatment of brain E_2 deficiencies (i.e. vasomotor hot flushes).

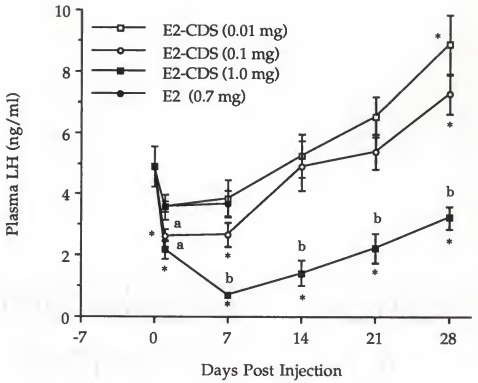


Figure 16. Dose and time-dependent effects of the E_2-CDS on plasma LH responses in ovariectomized rats. Animals received a single iv injection of the E_2-CDS on day 0 at doses of 0.01, 0.1 and 1.0 mg/kg bw. Also, the responses to an E_2 dose of 0.7 mg/kg, equimolar to the 1.0 mg/kg dose of E_2-CDS, is shown for day 1 and 7. Represented are means ± SEM for n = 7 rats per group per sampling time. The symbols indicate statistical differences as follows: *) different from vehicle group (day 0); a) different from 0.01 mg/kg; and b) different from both 0.01 and 0.1 mg/kg. The significance of interaction between factors (time and dose) was determined by two-way analysis of variance (ANOVA). The significance of differences among mean values at each dose level was determined over time by one-way ANOVA and Dunnett's test while the significance of differences among mean values of three dose levels (at each time point) was determined by one-way ANOVA and Scheffe F-test. The level of probability for all tests was $p<0.05$.

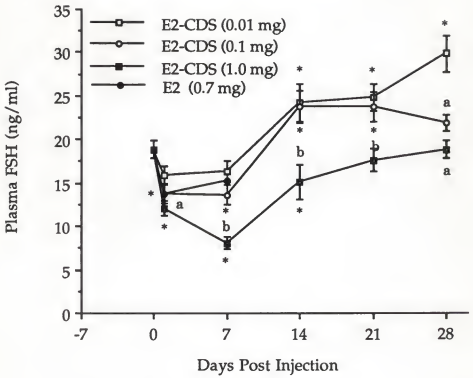


Figure 17. Dose and time-dependent effects of the E_2-CDS on plasma FSH responses in ovariectomized rats. Animals received a single iv injection of the E_2-CDS on day 0 at doses of 0.01, 0.1, and 1.0 mg/kg. Also, the responses to an E_2 dose of 0.7 mg/kg bw, equimolar to the 1.0 mg/kg dose of E_2-CDS, is shown for day 1 and 7. Represented are means ± SEM for n = 7 rats per group per sampling time. The symbols indicate statistical differences as follows: *) different from vehicle group (day 0); a) different from 0.01 mg/kg; and b) different from both 0.01 and 0.1 mg/kg. The significance of interaction between factors (time and dose) was determined by two-way analysis of variance (ANOVA). The significance of differences among mean values at each dose level was determined over time by one-way ANOVA and Dunnett's test while the significance of differences among mean values of three dose levels (at each time point) was determined by one-way ANOVA and Scheffe F-test. The level of probability for all tests was $p<0.05$.

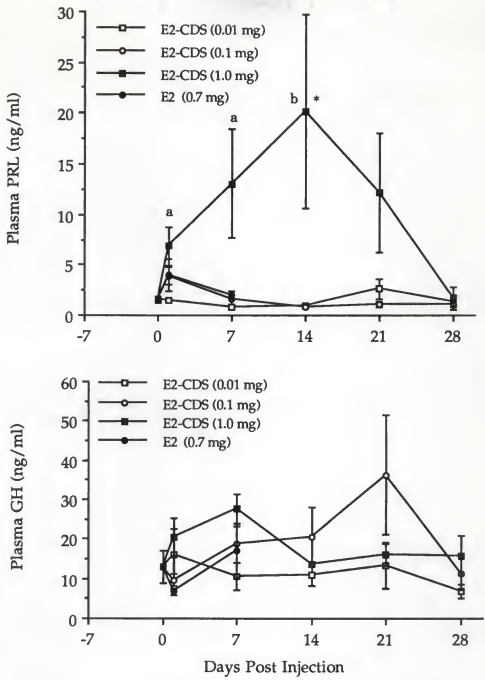


Figure 18. Dose and time-dependent effects of the E_2-CDS on plasma PRL (upper panel) and GH responses (lower panel) in ovariectomized rats. Animals received a single iv injection of the E_2-CDS on day 0 at doses of 0.01, 0.1 and 1.0 mg/kg or an E_2 dose of 0.7 mg/kg, equimolar to the 1.0 mg/kg dose of E_2-CDS, is shown for day 1 and 7 only. The symbols indicate statistical differences as follows: *) different from vehicle group (day 0); a) different from 0.01 mg/kg; and b) different from both 0.01 and 0.1 mg/kg.

Table 8: Dose and Time-Dependent Effects of the E_2-CDS on Peripheral Tissue Weights in Ovariectomized Rats

Tissue	Drug	Dose	Days after treatment				
		mg/kg	0	1	7	14	21	28
Anter. Pituit. (mg)	HPCD	---	11.8 ±0.5					
	E_2	0.7		13.5 ±0.7	12.8 ±0.7	ND	ND	ND
	E_2-CDS	0.01		12.0 ±0.8	13.1 ±0.5	14.4 ±1.5	11.9 ±0.8	13.0 ±0.6
	E_2-CDS	0.1		12.8 ±0.9	15.6* ±0.9	14.9* ±1.5	13.1 ±0.5	12.6 ±0.6
	E_2-CDS	1.0		13.3 ±0.9	19.0*,b ±1.0	20.7*,b ±1.3	16.2*,b ±1.0	15.0*,c ±0.6
Uterus (mg)	HPCD	---	154.2 ±8.6					
	E_2	0.7		261.4* ±13.0	182.9 ±24.0	ND	ND	ND
	E_2-CDS	0.01		183.8 ±12.3	179.1 ±29.0	196.4 ±42.0	121.5 ±7.0	107.8 ±8.0
	E_2-CDS	0.1		237.7* ±12.0	234.4* ±27.0	210.1* ±33.0	161.1 ±8.0	128.4 ±5.0
	E_2-CDS	1.0		281.4*,a ±20.0	427.0*,b ±31.0	372.9*,b ±28.0	267.4*,b ±31.0	235.4*,b ±16.0

Values are the mean tissue weights ± SEM. ND = Not determined.

* Different from time 0 values.

a Different from 0.01 mg/kg dose.

b Different from 0.01 and 0.1 mg/kg doses.

c Different from 0.1 mg/kg dose.

CHAPTER 8
EFFECTS OF THE E_2-CDS OR CASTRATION ON ANDROGEN AND ANDROGEN-DEPENDENT TISSUES IN MALE RATS

Introduction

Prostate carcinoma is the second most common cancer in males, and its incidence increases rapidly over the age of 50 (Silverberg & Lubera, 1986). Likewise, the development of benign prostatic hyperplasia also increases in incidence with age; however, there is no direct evidence that benign prostatic hyperplasia is a necessary prerequisite in the development of prostatic cancer (Berry et al., 1984; Rotkin, 1983). Despite the considerable clinical and scientific advances in the diagnosis, prognostication, and treatment, these prostatic diseases remain the second leading cause of morbidity and/or mortality in senescent males (Santen & English, 1989). Although epidemiological findings and sporadic reports of a familial occurrence of prostatic cancer suggest a genetic predisposition (Silverberg & Lubera, 1986), there is currently little evidence that this is a significant factor in most patients. A variety of other theories including neoplastic and endocrine metabolic factors have been proposed to explain the etiology and occurrence of prostatic adenocarcinoma and/or benign prostatic hyperplasia in men. Certainly, it is clear that the two major factors necessary for the genesis in men of these prostatic diseases are the presence of the testis and aging (Huggins & Hodges, 1941). Studies that attempt to correlate the incidence of the disease with age-related changes in testosterone levels or other endocrine factors have shown no consistent relationship as yet. However, there are

several lines of evidence that strongly support the possibility that prostatic growth/hyperplasia is dependent upon the endocrine activity of the testis: (1) prostatic maturation as well as prostatic hyperplasia does not occur in men who are castrated prior to puberty (Moore, 1944); (2) surgical castration (CAST) produces regression as well as beneficial effects toward the prostate hyperplasia of men (Cabot, 1896; Huggins & Hodges, 1941; White, 1895); (3) numerous studies have reported regression of prostatic hyperplasia with antiandrogens or estrogens treatment (Brendler, 1988; Foote & Crawford, 1988); and (4) recent biochemical studies have reported an association between prostatic hyperplasia with an abnormal accumulation of dihydrotestosterone (DHT), a potent testosterone (T) metabolite, in the human prostate (Isaacs et al., 1983). Whether the increase in the concentration of prostatic DHT is directly responsible for or is the result of the disease has not been completely resolved. However, further support for the DHT involvement comes from males with hereditary deficiency of the 5 α-reductase enzyme, an enzyme that converts T to DHT in androgen-target tissues (Imperato-McGinley et al., 1980, 1984). The lack of this enzyme results in very low levels of DHT as well as no palpable prostatic tissue, indicating that the prostate had not developed during embryogenesis.

Regarding the therapeutic aspects of the prostatic adenocarcinoma and/or hyperplasia, currently a variety of surgical and therapeutic means of inhibiting androgen production or blocking androgen action are being used (Labrie et al., 1983; Santen & English, 1989). These include surgical CAST, high-dose estrogen therapy, GnRH analogues, antiandrogens, and combination of low dose estrogens plus low dose antiandrogens. Each of these treatment regimens provides temporary benefit to patients, however, relapse usually occurs within a period of 1 to 2 years. Nevertheless, CAST or

high-dose estrogen therapy, the two conventional treatment paradigms, still remain as the treatment of choice for the endocrine-dependent management of prostatic cancer (van Steenbrugge et al., 1988). Both treatments are reported to be equally effective (i) in suppressing the circulating T levels (Carlstrom et al., 1989) and perhaps regression of the prostatic hyperplasia; and (ii) in controlling the symptoms of advanced prostatic cancer in 70-80% of patients with an average improved rate of survival of 5 years (Klein, 1979).

However, none of the two treatments is optimal or without major complication. For instance, high-dose estrogen therapy has been shown to cause severe cardiovascular complications (Henriksson & Edhag, 1986) and alterations in liver metabolism (von Schoultz et al., 1989). Estrogens, specially synthetic compounds, exert profound effects on liver-derived plasma proteins, coagulation factors, lipoproteins, and triglycerides when administered orally. However, most of the cardiovascular complications are the result of arterial ischemic events, and the majority of such events are the results of acute coronary arterial disease (Henrikson & Edhag, 1986). Recent studies have suggested that these dose-dependent liver-associated side effects and thus, cardiovascular complications may be reduced or even abolished when lowering the dose, provided that adequate T suppression is achieved (Jonsson et al., 1975; von Schoultz et al., 1989). Furthermore, numerous other reports also indicated that the marked interference with hepatic metabolism is associated with the kind of estrogen used (Ottosson, 1984; Ottosson et al., 1986). For example, the synthetic alkylated estrogen, ethinyl E_2, is a potent estrogen used in the treatment of prostatic cancer; however, it exerts profound effects on liver metabolism and thus, the use of a natural estrogen may be advantageous (Ottosson et al., 1986).

A potential problem associated with currently used estrogens is that these steroid hormones equilibrate among all body tissues due to their high lipophilicity. As a result, only a fraction of the administered estrogen dose accumulates at or near the intended site of action. Indeed, when these hormones are used therapeutically to specifically target the brain, the steroids must be given either frequently or in high doses in order to maintain therapeutically effective concentrations in the brain. Both of these treatment strategies lead to sustained increases in peripheral estrogen levels and, in particular, the liver is exposed to a greater drug burden. This is a major limiting factor in the use of these estrogenic products.

Based on the previous pharmacokinetic (Chapters 5 & 6) and pharmacodynamic (Chapter 7) observations and hence, the therapeutic potential of the E_2-CDS, we evaluated the effects of E_2-CDS (0.5 mg/kg b.w.) on androgen and androgen-responsive tissues in the present study. This dose of the E_2-CDS was chosen because a preliminary dose-response and time-course study indicated that 0.5 mg/kg is more effective than 1.0 mg/kg dose in suppressing androgen and androgen-responsive tissues in intact male rats.

Materials and Methods

Adult male Charles River (CD) rats (aged 3-4 months) used in this study were randomly divided into 9 experimental groups (7-8 rats per group). Two groups of rats were castrated while 7 other groups remained intact. CAST was performed by an abdominal incision under metofane anesthesia. Since surgical stress has previously been found not to affect the experimental parameters tested here, intact male rats used here were not subjected to sham operation.

To evaluate the effects of E_2-CDS or CAST on androgen and androgen-dependent sex accessory organs, a total of 5 different treatment conditions were evaluated: 1) intact control group; animals received no treatment; 2) CAST group; animals received no further treatment; 3) intact + E_2-CDS (x1) group; animals received a single iv injection (tail vein) of E_2-CDS (0.5 mg/kg bw); 4) intact + E_2-CDS (x2) group; animals received E_2-CDS (0.5 mg/kg bw, iv) once a week for 2 consecutive weeks; 5) intact + E_2-CDS (3x) group; animals received E_2-CDS (0.5 mg/kg bw, iv) once a week for 3 consecutive weeks. Animals were then sampled 7 or 14 days after the last treatment (treatment conditions 2 to 5).

Rats (7-8 per group) were sacrificed by decapitation either 7 or 14 days after the final administration of E_2-CDS or post CAST (treatment conditions 2-5). Immediately following decapitation, the trunk blood was collected in heparinized glass tubes on ice. The blood was centrifuged, and the plasma was separated and stored at -20°C for subsequent plasma hormone analysis. Also, the following tissues: prostate (the ventral lobe), seminal vesicles (both horns), testis, and anterior pituitary were rapidly dissected. The dissection of accessory sex organs was carried out according to previously reported guidelines (Lee, 1987). The ventral prostate was carefully grasped with forceps and dissected to the level of the ducts entering ventrally into the urethra. The seminal vesicles were retracted and expelled of seminal fluid. All tissues were blotted dry on paper then weighted to the nearest 0.1 mg.

Plasma LH, FSH, and PRL concentrations were measured in duplicate by RIA using NIDDK kits. Plasma LH, FSH, and PRL values are expressed as ng/ml of the LH-RP-2, FSH-RP-2 or the PRL-RP-3 reference standards, respectively, The intra-assay coefficients of variation were 3.37, 2.27, and 4.07% for LH, FSH, and PRL assays, respectively. Plasma samples containing

undetectable LH or FSH were assigned the respective assay sensitivity (0.25 for LH and 2.5 ng/ml for FSH). All the samples for each hormone were assayed in a single run.

Plasma E_2 and T concentrations were measured in duplicate by using Coat-A-Count Estradiol kits and Coat-A-Count Testosterone kits, respectively. The RIA sensitivity of the T assay was 0.2 ng/ml. The cross-reactivity of the T antibody to the DHT and E_2 has been reported to be 3.3 and 0.02%, respectively (technical information from Diagnostic Products).

Results

Effect of CAST or E_2-CDS on Plasma T Levels

Figure 19 shows the comparative plasma T concentrations of untreated control, post CAST and animals treated with E_2-CDS. CAST resulted in greater than 99% suppression of plasma T levels which remained suppressed at about the detection limit of the T assay for the time-course of study. Likewise, plasma T levels in E_2-CDS-treated animals were significantly suppressed by more than 96, 92, or 95% with 1, 2, or 3 injections, respectively, at 7 days after the last treatment. However, by 14 days after the last treatment with E_2-CDS, a 76, 82, or 91% reduction in plasma T was observed with 1, 2, or 3 injections, respectively. Even though plasma T levels began to gradually increase in a manner related to the number of E_2-CDS injections, T levels remained significantly suppressed compared with the control values.

Effect of CAST or E_2-CDS on Tissue Weights

The effects of CAST and E_2-CDS treatment on tissue weights are shown in Figures 20-23. CAST reduced the ventral prostate weights by more than 67% or 66% at 7 or 14 days after the orchidectomy, respectively (Figure 20). Treatment with E_2-CDS with all paradigms significantly reduced the ventral prostate weight to CAST level at 7 days after the last injection (Figure 20). The E_2-CDS-induced regression in ventral prostate weight remained significantly low at 14 days after the last treatment. Interestingly, the 3-injection paradigm of E_2-CDS was equivalent to CAST in reducing the ventral prostate weight by 62% at 14 days after the last injection.

Similarly, CAST significantly reduced the seminal vesicles weight by 52% or 63% at 7 or 14 days post CAST, respectively (Figure 21). Treatment of intact animals with E_2-CDS significantly reduced seminal vesicles weight by 44% to 62% and 34% to 55% at 7 and 14 days after the final treatment, respectively (Figure 21). The 3-injection paradigm of E_2-CDS was sufficient to reduce and chronically maintain the seminal vesicles weight at CAST level for the time-course studied.

Treatment of intact male rats with E_2-CDS had no significant effect on testes wet weight or wet weight/100 g bw (Figure 22). The 3-injection regimen of E_2-CDS caused about 19% reduction in testes weight at 7 or 14 days after the final injection (Figure 22).

Anterior pituitary weights were not changed by CAST (Figure 23). In contrast, pituitary weights increased significantly in a manner related to the number of injections in response to E_2-CDS administration (Figure 23).

Effect of CAST or E_2-CDS on Plasma Hormones

CAST significantly increased the plasma gonadotropins (LH and FSH) concentrations (Tables 9 & 10). By contrast, the plasma gonadotropin concentrations in animals treated with E_2-CDS were not significantly altered from control (Tables 9 & 10). Most likely, LH values in animals treated with E_2-CDS may have been significantly suppressed compared with control values, because more than 50% of LH values were below the detection limit of assay. However, since the sensitivity of LH assay was assigned as 0.25 ng/ml, statistically significant LH suppression was not achieved. A significant elevation in plasma PRL concentrations was observed only in animals treated with 3 injections of E_2-CDS (Table 9). Likewise, plasma E_2 concentrations were significantly elevated in animals treated with 2 and 3 injections of E_2-CDS at 7 days after the last injection (Table 9).

Discussion

The primary objective of endocrine therapy in prostate malignancy is the induction of an effective androgen deprivation, thus abolishing the growth promoting effects of androgens on the prostate tissue. These therapeutic objectives can be achieved by several mechanisms at different levels of the hypothalamo-pituitary-gonadal axis: 1) suppression of hypothalamic LHRH and hence, of pituitary LH release, thereby inhibiting T production by the testis; 2) surgical CAST which eliminates more than 90% of circulating T; 3) inhibition of androgen synthesis in the testis; and 4) blocking androgen action at the receptor site in the prostate. Thus, the choice of

treatment strategy largely relates to minimizing toxicity while optimizing the response rate as well as the duration of benefit.

In the present study, we described a newer endocrine approach in the treatment of androgen-dependent prostatic diseases. The evaluation of E_2-CDS effects on the normal androgen-responsive sex accessory organs with comparison to that of CAST demonstrated that: 1) the E_2-CDS at a single dose of 0.5 mg/kg was as effective as CAST in suppressing significantly the plasma T levels for 14 days after treatment; 2) the E_2-CDS treatment of intact male rats resulted in significant regression of the androgen-sensitive ventral prostate as well as seminal vesicle weight equivalent in magnitude to that of CAST alone; 3) interestingly, both the profound suppression of T levels and the prolonged duration of tissue regression, at 14 days after the final treatment, were observed even in the face of low plasma E_2 levels. Furthermore, these data suggest that the primary site of action where E_2 exerts its effects leading to T suppression is in the central nervous system. Previously, we have reported long-term suppression of LH in CAST male rats (Simpkins et al., 1986), stimulation of masculine sexual behavior in CAST male rats for 28 days (Anderson et al., 1987a), and long-term gonadotropin suppression in ovariectomized rats (Chapter 7) following a single iv administration of the E_2-CDS. These prolonged pharmacological effects of the E_2-CDS are consistent with the observations of the accumulation of E_2-Q^+, the oxidized form of the delivery system, in the brain with an apparent $t_{1/2}$ = 8-9 days in that tissue (Chapters 5 & 6). From this store of E_2-Q^+, E_2 is slowly released through non-specific hydrolysis of the carrier, resulting is sustained brain exposure to E_2.

The production of T by the Leydig cells of the testis are controlled via a negative feedback mechanism (Swerdloff, 1986). Increased levels of T exert a negative feedback on both the hypothalamus and the anterior pituitary, thus

inhibiting further LHRH and LH release. Recent studies suggest that E_2, the aromatized metabolite of T, is responsible in mediating the feedback effect of T on the hypothalamic neuronal system (Christensen & Clemens, 1974). Several lines of evidence have accrued to indicate that E_2 formation in the CNS is important for the effect of T. First, aromatization inhibitors decreased the effectiveness of T in inducing sexual behavior in CAST rats (Beyer et al., 1976). Second, intrahypothalamic implant of E_2 effectively restored the masculine sexual behavior in long-term CAST male rats (Christensen & Clemens, 1974). Finally, estrogen receptors and binding sites have been identified in the brain of male rats (Krey et al., 1980). Thus, prolonged exposure to estrogen hormone, whether by E_2-CDS or frequent administration of currently available estrogens, produces sustained suppression of hypothalamic LHRH secretion and hence, LH inhibition. Eventually, this leads to T deprivation or chemical castration.

Our results show that, like CAST, the E_2-CDS produced a significant T deprivation which remained suppressed for at least 14 days with a single injection, or for 28 days with 3 injections given once every 7 days for three consecutive weeks. Similarly, a single injection of E_2-CDS was sufficient to significantly regress both the ventral prostate and the seminal vesicles weights during the first week of treatment, while the 3 injections paradigm produced significant regression (equivalent to CAST) of these two tissues for at least 4 weeks. The profound suppression at 7 days of circulating T concentrations and/or of tissue weights (prostate and seminal vesicles) caused by the single or double injection of E_2-CDS, gradually began to recover at 14 days after the final treatment. However, these values remained significantly suppressed compared with the control values at 14 days after the last injection. Furthermore, unlike CAST, E_2-CDS treatment resulted in pituitary

hyperplasia. This time- and injection-related increase in pituitary weight appears to be due to the direct effects of E_2 on the pituitary gland. E_2 is well known to stimulate PRL secretion and to induce hyperplasia of lactotrophs (Chen & Meites, 1970). The source of E_2 responsible for the stimulation of anterior pituitary is most likely the brain E_2 and not the residual peripheral E_2. This can be explained by the anatomical relationship between the hypothalamus and the pituitary gland (Traystman, 1983).

We did not observe in intact male rats a statistically significant suppression in plasma gonadotropins (LH & FSH) in response to E_2-CDS treatment. However, plasma LH showed a progressive decline of 20 to 40% compared with the basal LH values. In fact, more than 50% of experimental animals treated with the E_2-CDS exhibited plasma LH values which were below the sensitivity of LH assay. Since plasma samples containing undetectable LH were assigned the assay sensitivity (0.25 ng LH/ml), statistically significant LH suppression was not obtained even if LH may have been significantly suppressed in animals that were treated with the E_2-CDS. Furthermore, since basal LH values of intact male rats were at the limit of the LH assay sensitivity, it was not possible to make a reliable correlation between the degree of LH suppression and tissue weight regression in rats treated with the E_2-CDS. Our previous observations in CAST rats showed that LH concentrations were suppressed by 82-90% for 4-12 days after a single injection of E_2-CDS (Simpkins et al., 1986). It should be noted, however, that CAST rats are much more sensitive than intact rats to the LH-suppressing effects of E_2-CDS.

Despite the apparent lack of significant LH suppression, plasma T levels were significantly suppressed by 96% or 76% at 7 or 14 days, respectively, after a single injection of E_2-CDS. This profound and sustained

suppression of circulating T levels were observed even in the face of low plasma E_2 levels, indicating the CNS involvement in mediating T suppression. Furthermore, when the dynamics of E_2-CDS effects on circulating T levels were compared with that of an equimolar dose of E_2 valerate, the E_2-CDS significantly suppressed T levels while E_2 valerate was ineffective (Anderson et al., 1989). Thus, as it has been consistently demonstrated (Anderson et al., 1987a,b, 1988a,b, 1989; Estes et al., 1987a,b, 1988; Simpkins et al., 1986, 1988, 1989a,b), the prolonged pharmacodynamic effects of E_2-CDS following a single injection are most likely due to "locking" of the E_2-Q^+ behind the BBB and there it serves as a brain depot for E_2.

Although, in the present study, we used a rat model to investigate the efficacy of E_2-CDS in reducing androgen levels with subsequent regression of the ventral prostate tissue, there are no conclusive data as to whether the rat prostate reflects a complete picture analogous to that of the aging human prostate. However, this model system has been employed for some years to gain further insight into the processes involved in the initiation and progression of prostatic hyperplasia (Pollard et al., 1989).

High-dose estrogens have also been reported to be effective in animal models of prostatic cancer (Daehlin & Damber, 1986). Recently, it was reported that E_2 implants (producing plasma E_2 levels as high as $4{,}442 \pm 962$ pmole/liter) to tumor-(prostatic carcinoma, PC-82) bearing mice resulted in tumor growth arrest with a subsequent decline of the tumor volume, which equals the effect of CAST (van Steenbrugge et al., 1988). Additionally, it was suggested that the effects of E_2 on the PC-82 tumor model were mainly indirect by suppressory effect on T secretion in the host animal, rather than a direct effect on the tumor tissue (van Steenbrugge et al., 1988). Conversely a number of studies demonstrated a direct action of estrogens at the cellular

level in the prostatic tissue (Daehlin et al., 1987). However, in spite of the possible direct effects of estrogen at the cellular level in addition to the indirect effects (CAST-like suppression of plasma T levels), the combined treatment of prostate cancer patients with CAST and estrogens did not improve survival when compared to either treatment alone (Blackard et al., 1973, 1975). Further, estrogens have also been shown to decrease 17α-dehydroxylase, 17 β-dehydrogenase and/or 17-20 desmolase (Kalla et al., 1980; Onoda & Hall, 1981) activity in the Leydig cells of the testis. Inhibition of these enzymes can increase the production of pregnenolone and progesterone and the concomitant decrease in T synthesis in Leydig cells.

In conclusion, because of the potential application of brain-enhanced estrogen delivery with sustained release in the brain as an alternative, we conducted this study. Collectively, the results support the concept that the E_2-CDS may be useful in the treatment of androgen-dependent prostatic hyperplasia. In comparison to the currently used estrogenic products, the E_2-CDS should achieve the sustained stimulation of brain E_2 receptors at lower doses or with less frequent dosing.

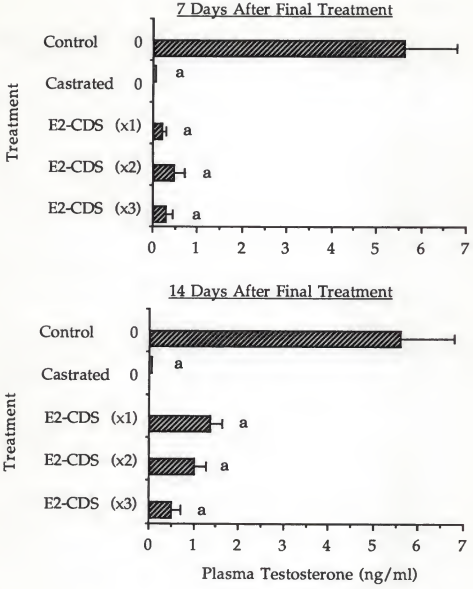


Figure 19. Effects of the E_2-CDS (0.5 mg/kg) or CAST on plasma testosterone levels either 7 days (upper panel) or 14 days (lower panel) after the final treatment. Animals received weekly iv (tail vein) injection of either the drug's vehicle (HPCD), or E_2-CDS (1 injection; x1), E_2-CDS (2 injections; x2), E_2-CDS (3 injections; x3), or were castrated (CAST). Represented are means ± SEM for n = 7-8 animals per group per sampling time. The symbol (a) denotes differences from control (HPCD)-treated animals as analyzed by ANOVA and Scheffe statistics.

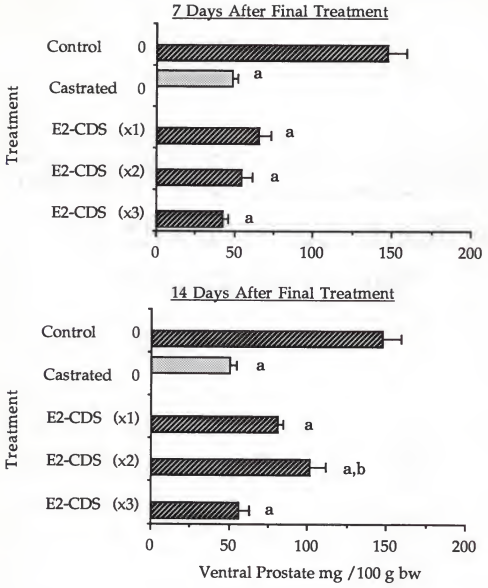


Figure 20. Effects of the E_2-CDS (0.5 mg/kg) or CAST on ventral prostate weight either 7 days (upper panel) or 14 days (lower panel) after the final treatment. Animals received weekly iv (tail vein) injection of either the drug's vehicle (HPCD), or E_2-CDS at a dose of 0.5 mg/kg (1 injection; x1), E_2-CDS (2 injections; x2), E_2-CDS (3 injections; x3), or were castrated (CAST). Represented are means ± SEM for n = 7-8 animals per group per sampling time. The symbol (a) denotes differences from control (HPCD)-treated animals and the symbol (b) indicates differences from CAST within the panel as analyzed by ANOVA and Scheffe statistics.

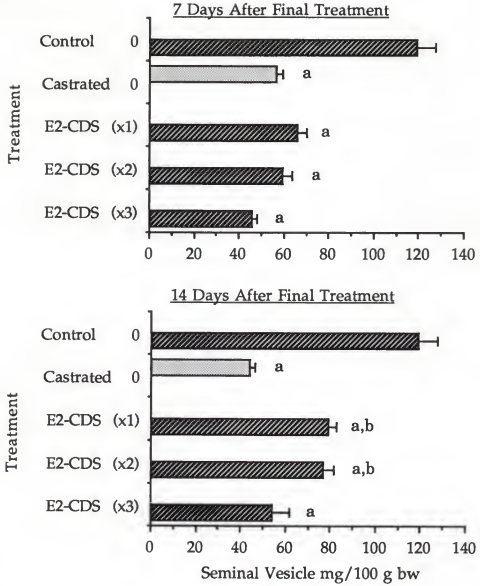


Figure 21. Effects of the E_2-CDS (0.5 mg/kg) or CAST on seminal vesicle weight either 7 days (upper panel) or 14 days (lower panel) after the final treatment. Animals received weekly iv (tail vein) injection of either the drug's vehicle (HPCD), or E_2-CDS at a dose of 0.5 mg/kg (1 injection; x1), E_2-CDS (2 injections; x2), E_2-CDS (3 injections; x3), or were castrated (CAST). The symbol (a) denotes differences from control (HPCD)-treated animals and the symbol (b) indicates differences from CAST within the panel as analyzed by ANOVA and Scheffe statistics.

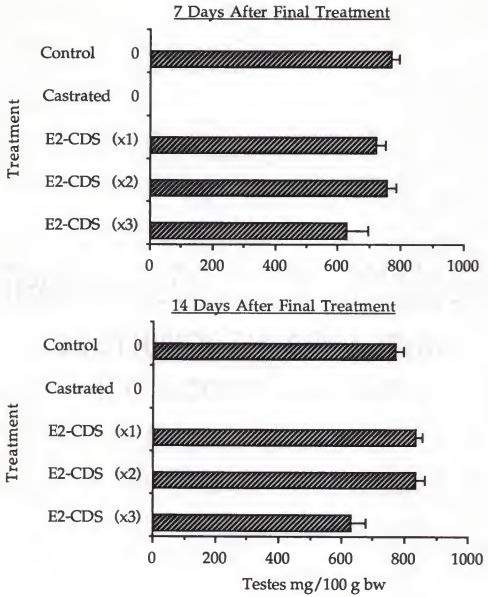


Figure 22. Effects of the E_2-CDS (0.5 mg/kg) or CAST on testis weight either 7 days (upper panel) or 14 days (lower panel) after the final treatment. Animals received weekly iv (tail vein) injection of either the drug's vehicle (HPCD), or E_2-CDS at a dose of 0.5 mg/kg (1 injection; x1), E_2-CDS (2 injections; x2), E_2-CDS (3 injections; x3), or were castrated (CAST). Represented are means ± SEM for n = 7-8 animals per group per sampling time.

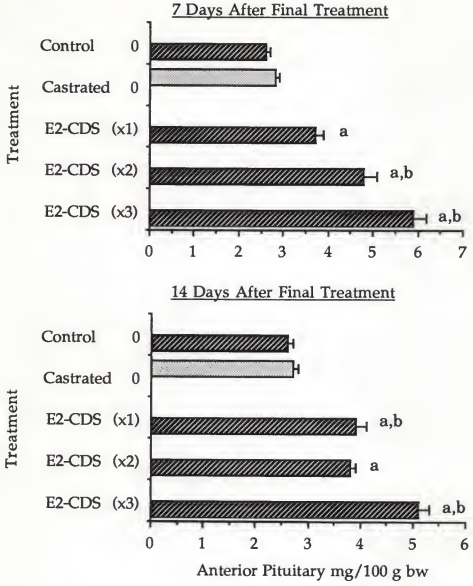


Figure 23. Effects of the E_2-CDS (0.5 mg/kg) or CAST on anterior pituitary weight either 7 days (upper panel) or 14 days (lower panel) after the final treatment. Animals received weekly iv (tail vein) injection of either the drug's vehicle (HPCD), or E_2-CDS at a dose of 0.5 mg/kg (1 injection; x1), E_2-CDS (2 injections; x2), E_2-CDS (3 injections; x3), or were castrated (CAST). Represented are means ± SEM for n = 7-8 animals per group per sampling time. The symbol (a) denotes differences from control (HPCD)-treated animals and the symbol (b) indicates differences from CAST within the panel as analyzed by ANOVA and Scheffe statistics.

Table 9: Effects of the E_2-CDS (0.5 mg/kg bw) or CAST on Plasma Hormone Concentrations at 7 Days after the Last Treatment in Male Rats.

Treatment Group	Inject. No.	E_2 (pg/ml)	E_2-Q+ (pg/ml)	LH (ng/ml)	FSH (ng/ml)	PRL (ng/ml)
Intact	-	12.3 ± 2.70	ND	0.50 ± 0.07	10.34 ± 1.88	28.15 ± 4.91
CAST	-	13.06 ± 2.85	ND	5.77 ± 0.42[a]	27.57 ± 1.00[a]	5.89 ± 1.06
E_2-CDS	1	40.45 ± 3.39	578.3 ± 46.9	0.30 ± 0.03[b]	6.65 ± 0.56[b]	35.97 ± 5.67
E_2-CDS	2	52.67 ± 7.69[a,b]	783.5 ± 90.9	0.28 ± 0.02[b]	6.53 ± 0.56[b]	43.21 ± 6.69
E_2-CDS	3	70.63 ± 18.29[a,b]	654.8 ± 54.4	0.39 ± 0.07[b]	7.33 ± 0.98[b]	80.64 ± 12.88[a,b]

ND Not determined
a Different from Intact
b Different from CAST

Table 10: Effects of the E_2-CDS (0.5 mg/kg bw) or CAST on Plasma Hormone Concentrations at 14 Days after the Last Treatment in Male Rats.

Treatment Group	Inject. No.	E_2 (pg/ml)	E_2-Q+ (pg/ml)	LH (ng/ml)	FSH (ng/ml)	PRL (ng/ml)
Intact	-	12.3 ± 2.70	ND	0.50 ± 0.07	10.34 ± 1.88	28.15 ± 4.91
CAST	-	7.30 ± 1.87	ND	8.15 ± 0.55[a]	26.57 ± 1.34[a]	4.84 ± 0.82
E_2-CDS	1	20.07 ± 4.11	190.2 ± 22.9	0.27 ± 0.01[b]	10.53 ± 0.91[b]	38.34 ± 9.96
E_2-CDS	2	13.73 ± 4.31	120.9 ± 18.8	0.27 ± 0.02[b]	12.00 ± 1.86[b]	15.93 ± 3.10
E_2-CDS	3	30.01 ± 4.17	131.5 ± 20.4	0.31 ± 0.03[b]	11.83 ± 1.30[b]	12.47 ± 3.19

ND Not determined
a Different from Intact
b Different from CAST

CHAPTER 9
EFFECTS OF THE E_2-CDS OR E_2 PELLET ON TAIL-SKIN TEMPERATURE RESPONSES IN OVARIECTOMIZED FEMALE RATS

Introduction

Over the past 50 years, numerous clinical studies in either natural or ovariectomy-induced menopausal women have provided evidence that gonadal steroid withdrawal causes alterations in the central thermoregulatory system which then leads to vasomotor hot flushes (Casper & Yen, 1985; Lauritzen, 1973; Yen, 1977). After menopause or ovariectomy, the decreasing production of ovarian estrogens/progestins leads abruptly to a number of central nervous system (CNS)-mediated steroid-withdrawal symptoms (Casper & Yen, 1985; Lauritzen, 1973; Yen, 1977). These symptoms most often express themselves as hot flushes, perspiration, depression, anxiety, changes in memory, headaches, insomnia and irritability (Clayden et al., 1974; Meldrum et al., 1979; Paterson, 1982). However, their intensity and frequency vary among women and symptoms do not occur at all in about 15 to 25% of menopausal women (Casper & Yen, 1985). The most frequent symptom of the menopause is the hot flush, an episodic disturbance of thermoregulation characterized by a sensation of heat followed by a sudden spreading of flush and perspiration (Casper & Yen, 1985; Clayden et al., 1974; Lauritzen, 1973; Meldrum et al., 1979; Paterson, 1982; Yen, 1977). These physiological alterations appear to be the result of autonomic discharge which causes peripheral vasodilation and heat loss, with a considerable drop in core body temperature (Nesheim & Saetre, 1982). Furthermore, each flush is preceded

by an acceleration in heart rate (Molnar, 1975) and a surge in secretion of luteinizing hormone (LH) accompanies the flush response (Casper et al., 1979; Meldrum, 1979). Additionally, flushes can be provoked by warm ambient temperature, hot drinks, alcoholic beverages, mental stress, and hypoglycemia (Simpkins & Katovich, 1984).

Although the mechanism(s) involved in the menopausal syndrome is as yet unknown, numerous clinical and experimental studies have implicated the hypothalamic noradrenergic system (Casper & Yen, 1985; Simpkins & Katovich, 1984), luteinizing hormone-releasing hormone (LHRH) neuronal system (Gambone et al., 1984), and endogenous opioid peptide system (Casper & Yen, 1985; Simpkins & Katovich, 1984) in its genesis. The evidence that gonadal steroid hormones modulate or influence the activity of each of these central hypothalamic neuronal systems is compelling (McEwen et al., 1984; McEwen & Parsons, 1982; Roselli & Resko, 1990). Biochemical, autoradiographic and immunohistochemical experiments have demonstrated that the most dense collections of cells containing gonadal steroid hormones and their receptors in the rat brain are found in the medial preoptic area, hypothalamus, and in the limbic system structures (Luine et al., 1975; Morrel et al., 1975; Pfaff & Keiner, 1973). These brain regions are key elements in the neural circuits that regulate the neuroendocrine events of reproduction and behavior (Barraclough & Wise, 1982; Goodman & Knobil; Plant, 1986; Christensen & Clemens, 1974). A plausible hypothesis currently held for the mechanism of the hot flush states that normally the opioidergic-noradrenergic-LHRH neuronal systems are serially involved in coupling or mediating the regulatory (feedback) influence of gonadal steroids on LH secretion and perhaps thermoregulation (Casper & Yen, 1985). However, in postmenopausal, as well as in long-term ovariectomized women, this neural

circuit, in particular the opioid neurons/receptors component, is uncoupled from the LH release mechanism (Casper & Yen, 1985; Simpkins & Katovich, 1984). Hence, replacement of gonadal steroids in postmenopausal women would exert a stabilizing influence on these neuronal network or the mechanisms responsible for the flush response. Nevertheless, the mechanism(s) through which gonadal steroids exert their stabilizing effects remains unknown.

Although replacement therapy with estrogens and/or progestins has been shown to be effective in most patients in alleviating the symptoms of the menopause (Campbell & Whitehead,1977; Casper & Yen, 1985; Lauritzen, 1973; Upton, 1984; Yen, 1977), numerous retrospective studies indicated an increased risk of peripheral toxicities, including the risk of breast and endometrial cancer (Bergkvist et al., 1988; Berkowitz et al., 1985; Ettinger et al., 1988; Persson, 1985), cardiovascular complications (Barrett-Connor et al., 1989; Kaplan, 1978; Thomas, 1988), and alteration in hepatic metabolism (Burkman, 1988). The potential problem associated with currently used steroids medication is that these hormones equilibrate among all body tissues due to their high lipophilicity. As a result, only a fraction of the dose accumulates at or near the site of action in the brain. Furthermore, steroid receptors are present in many peripheral tissues creating the potential of untoward peripheral site effects (Walters, 1985).

Given the aforementioned limiting factors in estrogen replacement therapy, the preferential brain delivery of E_2 with the E_2-CDS may offer an effective treatment strategy for postmenopausal symptoms by providing sufficient E_2 to the brain while avoiding peripheral toxicities.

This study was undertaken to assess the effects of E_2-CDS on the rise in tail-skin temperature (TST) in the morphine-dependent, naloxone-

withdrawal rat model developed in our laboratory (Simpkins & Katovich, 1984). This animal model was originally developed to study the neuroendocrine mechanism(s) leading to the flush response as well as evaluating new and existing drugs for their efficacy in treating the menopausal symptoms. We have previously demonstrated that naloxone-induced withdrawal in the morphine-dependent rat model results in a surge in TST that is preceded by tachycardia, accompanied by hypersecretion of LH, and is followed by a significant fall in core body temperature (Simpkins & Katovich, 1984). Each of these responses is similar in both magnitude and duration and temporally associated to those observed in menopausal hot flushes (Simpkins et al., 1983).

Materials and Methods

To evaluate the effects of E_2-CDS on TST responses, adult female rats were bilaterally ovariectomized (OVX) under metofane anesthesia and experiments were initiated 2 weeks after ovariectomy. On day 15 after ovariectomy, rats were randomly divided into 4 groups (7-8 rats per group). These experimental groups were designated and treated as follows: 1) OVX + HPCD control, animals in this group received weekly iv injection of HPCD (vehicle) for 3 weeks; 2) OVX + E_2 pellet, these animals were subcutaneously (sc) implanted (under light metofane anesthesia) with an E_2 pellet weekly for 3 weeks (removing the old pellet before implanting a new one); 3) OVX + E_2-CDS multiple injections, these animals received weekly iv injection of E_2-CDS at a dose of 1.0 mg/kg b.w. for 3 weeks; and 4) OVX + E_2-CDS single injection, these animals received a single iv injection of E_2-CDS at 1 week before testing.

Morphine dependency was produced after initiation of estrogen treatment as described previously (Katovich & O'Meara 1986; Simpkins & Katovich, 1984). Briefly, one morphine pellet, containing 75 mg morphine free base, was sc implanted at 17 days (long-term treatment, groups 1-3), or at 3 days (short-term treatment, group 4) after initiation of estrogen treatment. Two days after the first morphine pellet, two additional morphine pellets were sc implemented. This regimen of morphine treatment has been utilized in our laboratory (Simpkins et al., 1983; Simpkins & Katovich, 1984) to consistently produce typical symptoms of morphine dependency, tolerance, and withdrawal (Wei et al., 1973). Four days after the initiation of morphine treatment, on the morning of the twenty first day (long-term treatment) or the seventh day (short-term treatment) after initiation of estrogen treatment, animals were lightly restrained in wire mesh tunnel cages with a wooden floor. TST was measured with a copper-constantan thermocouple that was taped to the dorsal region of the tail at approximately 2 cm from its base so that the thermocouple contacted the skin near the base of the tail. Rectal temperature (RT) was measured with a copper-constantan thermocouple inserted 6 cm beyond the anus and taped to the base of the tail. TST and RT were recorded at 2-min intervals. Rats were allowed 1 hr to acclimate to the restraining cages while control measurements were recorded. At the end of the control period, rats were administered naloxone HCl (0.5 mg/kg b.w., sc). TST and RT were recorded for an additional 90 min in a room maintained at 24 ± 1°C. At the conclusion of the temperature study, all animals were killed by decapitation and the trunk blood was collected in heparinized tubes. The blood was centrifuged and the plasma separated and stored at -20°C until hormone analysis.

Our previous studies (Simpkins et al., 1983; Simpkins & Katovich, 1984) demonstrated that implants of placebo followed by naloxone administration as well as implants of morphine which followed by saline injection did not induce TST surge in these treatment groups. Therefore, these control groups were not repeated again in the present study.

Plasma LH, FSH, and PRL concentrations were measured in duplicate by RIA using NIDDK kits. Plasma LH, FSH, and PRL values are expressed as ng/ml of either the LH-RP-2, FSH-RP-2 or the PRL-RP-3 reference standards, respectively. The intra-assay coefficients of variation were 4.19%, 3.32%, and 4.4% for LH, FSH, and PRL assays, respectively. Plasma E_2 concentrations were measured in duplicate by the RIA employing Coat-A-Count Estradiol kits. All the samples for each hormone were assayed in a single run.

Results

There was no significant effect of estradiol on the basal TST (Figure 24; Table 11) or the basal RT (Figure 25; Table 11) in the morphine-dependent animals. However, administration of naloxone (0.5 mg/kg) to the morphine-dependent rats resulted in a rapid increase in TST and a subsequent decline in RT (Figures 24 & 25; Table 11). The mean maximal elevation in TST were 6.4 $\pm$ 0.2, 6.4 $\pm$ 0.1, 3.4 $\pm$0.6, and 4.9 $\pm$ 0.5°C in the HPCD, E_2 pellet, multiple E_2-CDS, and single E_2-CDS groups, respectively (Table 11). Multiple injections of the E_2-CDS resulted in significant attenuation (more than 47%) of the naloxone-induced maximal rise in TST (Figure 24; Table 11). A single injection of the E_2-CDS for 7 days also attenuated the maximal rise in TST by 25%, but this treatment effect was not statistically significant. By contrast, treatment with 17 β-E_2 (E_2 pellet regimen) had no effect on the surge of TST (Figure 24; Table

11). The naloxone-induced surge in TST returned to normal range at the end of 70 to 90 min of study.

The area under the TST curve (AUC) was significantly reduced by 42% or 46% with multiple injections of E_2-CDS compared with the HPCD control or the E_2 pellet, respectively. Likewise, treatment with a single injection of E_2-CDS reduced the AUC by 13% or 20% compared to the HPCD group or the E_2 pellet, respectively, however these effects were not statistically significant.

The mean maximal decline in RT was 2.3 ± 0.3, 3.1 ± 0.4, 3.3 ± 0.3, and 2.5 ± 0.4°C in the HPCD, E_2 pellet, multiple E_2-CDS, or single E_2-CDS groups, respectively (Figure 25). Estrogen treatment with E_2-CDS or E_2 pellet had no significant effect on Rt. The naloxone-induced decline in RT returned to normal at 3 to 4 hrs after naloxone administration (data not shown).

Plasma E_2 concentrations were significantly elevated with the E_2-pellet as well as with the multiple E_2-CDS treatment (Table 12). However, the E_2-pellet treatment produced plasma E_2 concentrations which were significantly higher (2- to 6- fold) than those produced by single or multiple E_2-CDS treatment (Table 12).

Plasma gonadotropin concentrations (LH and FSH) were significantly suppressed relative to HPCD control with the E_2-pellet as well as the single and multiple E_2-CDS treatment (Table 12).

Plasma PRL concentrations in animals treated with either the E_2 pellet or the multiple E_2-CDS were significantly elevated relative to PRL levels of the HPCD group or the single-injected E_2-CDS group. Furthermore, the magnitude of PRL stimulation with the E_2-pellet treatment was 1.5- and 3.5-fold greater than that of multiple- or single-injected E_2-CDS, respectively (Table 12).

Discussion

In this study, we used the morphine-dependent, naloxone-withdrawal rat model to evaluate the effectiveness of E_2-CDS for the treatment of hot flushes. To our knowledge, this is the only animal model (Simpkins & Katovich, 1984) available to evaluate effectively alternative therapies for this common disease.

The results of this study for the first time demonstrate that: (1) the E_2-CDS can attenuate significantly the naloxone-induced surge in TST of the morphine-dependent rats; (2) 17 β-E_2 pellet treatment has no effect on naloxone-induced rise in TST; and (3) the significant effects of E_2-CDS are achieved at low plasma E_2 concentrations. Furthermore, these data support the hypothesis that the primary site of action where E_2 exerts its stabilizing effects on the mechanism(s) which control thermoregulation is in the CNS and not in the periphery (Casper & Yen, 1985; Lauritzen, 1973; Yen, 1977). This is clearly shown by the fact that treatment with 17 β-E_2 pellet which produced superphysiological plasma E_2 (2-fold greater than those produced by the multiple injections of E_2-CDS) did not exert any stabilizing effect on the rise of TST. These pellets were shown in our laboratory to release high concentrations of E_2 (280-180 pg/ml) in plasma on day 1 and 2 after implantation. However, between days 5 and 14 of implantation stable levels of E_2 (100-80 pg/ml) were observed. Contrary to our findings in the present study, 17 β-E_2 has recently been reported to produce significant attenuation in the magnitude of the flush response using the same animal model (Katovich & O'Meara, 1986). The reason for this discrepancy seems to be the result of very high concentrations of plasma E_2 in the previous study. Utilizing E_2

pellet (Katovich & O'Meara, 1986) which produced very high plasma E_2 concentrations (7-fold greater than E_2 concentrations produced by E_2 pellet in the present study), would certainly lead to higher brain E_2 levels. Therefore, maintaining therapeutically effective E_2 levels in the brain will eventually produce brain-mediated E_2 effect. Nevertheless, estrogens or estrogen-esters are used therapeutically in postmenopausal patients (Campbell & Whitehead, 1977; Casper & Yen, 1985; Lauritzen, 1973; Upton, 1984; Yen, 1977), and these agents are effective in alleviating menopausal symptoms (Campbell & Whitehead, 1977; Paterson, 1982; Upton, 1984). Unfortunately, these estrogenic products are administered either in frequent doses, or as a large depot form, to achieve and maintain therapeutically effective levels in the brain. Both of these treatment strategies lead to sustained increases in peripheral estrogen levels which have been shown in numerous studies to increase peripheral toxicities (Barrett-Conner et al., 1989; Burkman, 1988; Campbell & Whitehead,1977; Casper & Yen, 1985; Kaplan, 1978; Lauritzen, 1973; Thomas, 1988; Trapido et al., 1984; Upton, 1984).

Our previous observations, including the E_2-CDS kinetics in intact male rats (Chapter 5) and in OVX rats (Chapter 6) as well as the pharmacodynamic effects in OVX rats (Chapter 7), long-term suppression of gonadotropin secretion in CAST male rats (Simpkins et al., 1986), long-term suppression of androgen secretion and weights of androgen-responsive tissues (Chapter 8), stimulation of masculine sexual behaviors in CAST male rats for 28 days (Anderson et al., 1987a) following a single iv administration of the E_2-CDS and our present observation, that E_2-CDS but not 17 β-E_2 significantly attenuates the naloxone-induced rise in TST, provide strong support for the proposed mechanism of the E_2-CDS. That is, following E_2-CDS administration, the delivery system undergoes rapid oxidation

providing the basis for "locking" the intermediate metabolite, E_2-Q+, behind the BBB, there it serves as a brain depot for E_2. From this store of E_2-Q+, E_2 is slowly released through non-specific hydrolysis of the carrier, resulting in sustained brain exposure to E_2.

Estrogen treatment of OVX morphine-dependent rats with both the E_2-CDS and E_2 pellet resulted in significant suppression of gonadotropins (LH and FSH) secretion. These effects of E_2-CDS on gonadotropins are consistent with the previously reported pharmacodynamic behaviors of the delivery system (Anderson et al., 1987a,b, 1988a,b, 1989; Sarkar et al., 1989; Simpkins et al., 1986, 1988, 1989a,b). The fact that the magnitude of LH suppression was greater with E_2-CDS treatment in the face of lower plasma E_2 concentrations, indicate that the prolonged and sustained inhibitory effects of E_2-CDS are due primarily to sustained suppression of LHRH secretion from the hypothalamus (Sarkar et al., 1989). Sarkar et al. (1989) have reported reduction in LHRH release into the hypophyseal portal system and no change in pituitary responsiveness to LHRH following E_2-CDS treatment.

An important question is whether morphine withdrawal in the rat is analogous to the postmenopausal flushes. The remarkable similarities between the symptoms of opiate withdrawal and the postmenopausal syndrome (Simpkins et al., 1983) suggest a common underlying neuronal mechanism(s) mediating these changes in both the addicted rats and the postmenopausal women (Casper & Yen, 1985; Simpkins & Katovich, 1984). Our observations that E_2-CDS treatment in some animals (50% or less) did not completely stabilize these underlying mechanism(s) in order to prevent the flush response may argue against this animal model. This heterogeneity in response to estrogen replacement therapy, however, is observed in postmenopausal women as well. That is, first of all, the intensity and

frequency of the postmenopausal symptoms vary among women, and symptoms do not occur at all in about 25% of postmenopausal women (Casper & Yen, 1985). Also, it is important to note that not all postmenopausal women on hormone replacement therapy respond positively to the steroid medications (Paterson, 1982), even though it is generally believed that the flushing response is due to steroid-hormone withdrawal phenomenon (Casper & Yen, 1985; Lauritzen, 1973; Simpkins & Katovich, 1984; Yen, 1977).

It is also important to point out that the length of exposure to E_2 via E_2-CDS may be as important as the dose administered to the morphine-dependent rats. Perhaps the variability or heterogeneity in response is due to the fact that all animals may eventually respond to E_2-CDS treatment, but it may take variable durations of sustained E_2 exposure via E_2-CDS treatment in order to observe an attenuation in TST response.

In conclusion, these results support the view that the E_2-CDS may be potentially useful in the treatment of brain E_2 deficiencies (i.e., vasomotor hot flushes). Further experimental as well as clinical investigations pertaining to the therapeutic efficacy of the E_2-CDS in this regard is warranted. In comparison to the currently used estrogenic products, the E_2-CDS should achieve the sustained stimulation of brain E_2 receptors at lower doses or with less frequent dosing.

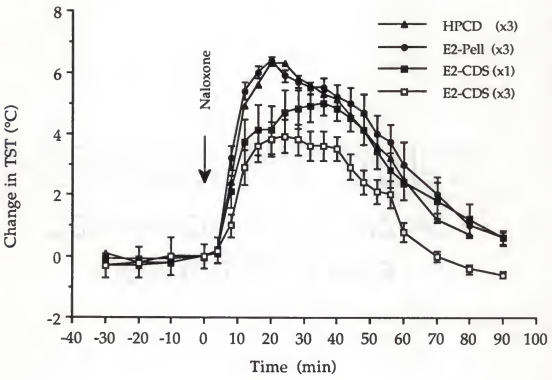


Figure 24. Effects of the E_2-CDS or E_2 pellet on the mean TST responses induced by naloxone administration (0.5 mg/kg; sc) to morphine-dependent ovariectomized rats. Animals were treated weekly with either the vehicle (HPCD, x3, 3 injections total over 3 weeks), E_2-CDS (1.0 mg/kg, x3, 3 injections total over 3 weeks), E_2 pellet (0.5 mg, x3, 3 implants total over 3 weeks), or E_2-CDS (1.0 mg/kg, x1, 1 injection for 1 week). Morphine dependency was produced after initiation of estrogen treatment. Four days after the initiation of morphine treatment, on the morning of the 21st day (long-term) or the 7th day (short-term) after the initiation of estrogen treatment, animals were lightly restrained in wire mesh cages. TST was then recorded at 2-min intervals. Represented are the means ± SEM for n=7-8 rats per group.

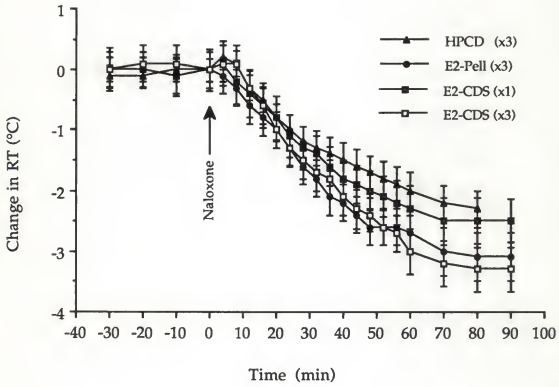


Figure 25. Effects of the E_2-CDS or E_2 pellet on the mean RT responses induced by naloxone administration (0.5 mg/kg; sc) to morphine-dependent ovariectomized rats. Animals were treated weekly with either the vehicle (HPCD, x3, 3 injections total over 3 weeks), E_2-CDS (1.0 mg/kg, x3, 3 injections total over 3 weeks), E_2 pellet (0.5 mg, x3, 3 implants total over 3 weeks), or E_2-CDS (1.0 mg/kg, x1, 1 injection for 1 week). Morphine dependency was produced after initiation of estrogen treatment. Four days after the initiation of morphine treatment, on the morning of the 21st day (long-term) or the 7th day (short-term) after the initiation of estrogen treatment, animals were lightly restrained in wire mesh cages. RT was then recorded at 2-min intervals. Represented are the means ± SEM for n=7-8 rats per group.

Table 11: Effects of the E_2-CDS or E_2 pellet on Basal Temperature, Maximal Change in TST, and Area Under the 90 Min TST Curve in Ovariectomized, Morphine-Dependent Rats

Treatment Group		Basal RT	Basal TST	Max Δ TST	AUC
		----------(°C)----------			(°C-Min)
HPCD	(3)*	39.8 ± 0.2	26.4 ± 0.1	6.4 ± 0.2	335.5 ± 24.9
E_2 Pellet	(3)	38.7 ± 0.2	26.2 ± 0.1	6.4 ± 0.1	361.8 ± 19.4
E_2-CDS	(3)	39.1 ± 0.3	26.9 ± 0.4	3.4 ± 0.6[a,b]	197.8 ± 35.3[a,b]
E_2-CDS	(1)	38.6 ± 0.3	26.5 ± 0.1	4.9 ± 0.5	291.5 ± 32.8

* Number of injection(s)/pellet(s)
a Different from HPCD group
b Different from E_2-pellet group

Table 12. Effects of the E_2-CDS or E_2 Pellet on Plasma Hormone Concentrations in OVX, Morphine-Dependent Rats.

Treatment Group	E_2	E_2-Q+	LH	FSH	PRL
	(pg/ml)	(pg/ml)	(ng/ml)	(ng/ml)	(ng/ml)
HPCD (3)*	3.67 ± 0.95	ND	10.11 ± 2.77	40.53 ± 3.24	15.49 ± 9.93
E_2 Pellet (3)	89.80 ± 9.97[a,b]	ND	0.90 ± 0.19[a]	13.26 ± 1.23[a,c]	960.50 ± 100.4[a,c]
E_2-CDS (3)	44.10 ± 6.37[a,c]	866.66 ± 53.42[c]	0.43 ± 0.08[a]	12.16 ± 1.69[a,c]	677.64 ± 86.72[a,c]
E_2-CDS (1)	13.45 ± 3.53	224.12 ± 17.88	0.57 ± 0.09[a]	20.43 ± 1.06[a]	279.50 ± 96.85

ND Not determined
* Number of injection(s)/pellet(s)
a Different from HPCD group
b Different from E_2-CDS(3) or E_2-CDS(1) group
c Different from E_2-CDS(1) group

CHAPTER 10
GENERAL DISCUSSION

The studies in this dissertation evaluated the pharmacokinetics and pharmacodynamic consequences of the redox-based estradiol-chemical delivery system (E_2-CDS) for the brain in the rat. The synopses of the major findings drawn from these studies are (1) development of a technique for the simultaneous quantitation of E_2-Q^+ and E_2 that is reliable, sensitive and applicable to a wide variety of tissues (Chapter 4; Rahimy et al., 1989a); (2) documentation of the preferential deposition and retention of the E_2-CDS metabolites, E_2-Q^+ and E_2, in the CNS tissue with a $t_{1/2}$ = 8-9 days in male (Chapter 5; Rahimy et al., 1988, 1990a) and in OVX female rats (Chapter 6; Rahimy et al., 1990b); (3) demonstration of the relatively rapid disappearance of these metabolites from various peripheral tissues (Chapters 5 & 6; Rahimy et al., 1988, 1990a,b); (4) determination of the prolonged suppression of gonadotropin secretion LH and FSH in OVX female rats in a dose- and time-dependent manner (Chapter 7; Rahimy et al., 1989b, 1990c); (5) demonstration of the sustained suppression of T secretion and weight of androgen-responsive tissues equivalent in magnitude to that of castration level (Chapter 8); and (6) demonstration of significant attenuation of the naloxone-induced surge in TST of morphine-dependent rats in the face of very low plasma E_2 levels (Chapter 9).

The formidable task of delivering the drugs of choice to the CNS has long been recognized particularly by neuropharmacologists. This is because of the unique feature of the brain, the BBB, that allows only lipophilic agents to

gain access to the CNS. As a result, many potentially useful therapeutic agents, i.e. water soluble drugs are excluded from entering the brain. To overcome this barrier and thus, to enhance CNS drug concentration, various strategies for drug flux through the BBB have been developed. These include: (a) physical approaches, i.e. intraventricular/intrathecal infusion and implantable pump (Hammond, 1988); and (b) chemical approaches, i.e. prodrugs (Bodor, 1981, 1985, 1987; Sinkula & Yalkowsky, 1975; Stella, 1975), liposomes (Weiner et al., 1989), and the use of membrane transport systems to deliver nontransportable peptides through the BBB (Pardridge, 1986).

The intraventricular administration of drugs, in addition to being an invasive technique, is inefficient in cases where the drugs of choice are polar and highly water soluble. For instance, the intraventricular administration of polar drugs results in uneven or incomplete distribution in the brain since these agents are solubilized primarily in the aqueous compartment (CSF). Furthermore, this approach tends to bath the surface of the brain, perhaps because the efflux of the drug out of the ventricles and into the superior sagittal sinus is much faster than diffusion into the brain parenchyma (Pardridge, 1988a).

A general, more practical approach to increase brain concentrations of water soluble drugs and thus their therapeutic efficacy has been the design of prodrug formulation (Bodor, 1981, 1985; Sinkula & Yalkowsky, 1975; Stella, 1975). The purpose of prodrug modification is to increase the concentration of the active drug at or near its site of action, thereby increasing its efficacy. However, by increasing the lipophilicity of a drug nonspecifically via the prodrug approach, it may not only enhance its diffusion through the BBB , but also enables the uptake of the compound into all other tissues and thus, exposure to a greater drug burden. This method of nonselectivity of drug

delivery is the major limiting factor in the prodrug design, specially those with cytotoxicity or those with broad spectrum of peripheral sites of action such as steroids.

A more recent, pharmacologic-based strategy which has shown great potential for drug delivery is the liposome preparation (Weiner et al, 1989). An assortment of drugs, including peptide and protein compounds, may be incorporated in the liposomes, which can then be administered by different routes. The physicochemical properties of the liposomes allows the encapsulation of a drug molecule either in the aqueous space or intercalation into the lipid bilayer matrix, depending on the properties of drugs. Liposomes are, however, taken up by cells lining the reticuloendothelial system, and do not appear to be useful for drug delivery through the BBB. The paradoxical reason for this may be the larger size of these vesicles which prevent them from crossing the BBB via lipid-mediated transport mechanisms even though liposomes are highly lipid soluble (Pardridge, 1988a).

The most highly developed and promising strategy for improving drug delivery through the BBB is the coupling of water- or lipid-soluble drugs to the redox-based dihydropyridine nucleus (Bodor, 1987; Bodor & Brewster, 1983; Bodor et al., 1981). This strategy for the CNS drug delivery offers several advantages. First, the application of the carrier, dihydropyridine, to a drug increases the lipid solubility of the drug, because of highly lipid-soluble nature of the carrier. Second, the carrier exhibits an intermediate enzymatic oxidation to a quaternary pyridinium ion, which encourages preferential brain deposition and retention by "locking" the charged, oxidative metabolite behind the BBB; enzymatic hydrolysis of the charged-pyridinium drug complex in a subsequent step provides sustained release of the parent drug in

the brain. Finally, the carrier system simultaneously enhances the rate of elimination of the drug, specially if lipoidal in nature, in an inactive form, from peripheral tissues following the oxidation to a hydrophilic quaternary form.

The present work evaluated this redox-based chemical-delivery approach to brain estrogen delivery. To determine reliably the tissue distribution and thus to document the effectiveness of E_2-CDS, a specific and sensitive method was essential (Chapter 4; Rahimy et al., 1989a). Such criteria are extremely important, particularly for the E_2-CDS, since the E_2-CDS metabolite, E_2, is not only present in low concentration but also active at low pg/g tissue. Furthermore, the intermediate metabolite, E_2-Q+, is present in concentrations much higher than E_2. Thus, to accurately quantitate E_2 levels in tissue samples, the assay method must be capable of distinguishing low levels of E_2 in the presence of high concentrations of E_2-Q+. These problems were resolved by employing and optimizing the reproducibility of an RIA procedure for E_2 determination in all tissues and fluid (Chapter 4). The RIA provides the needed sensitive end point (0.8 to 1.2 pg/assay tube) as well as the required specificity (cross-reactivity of <0.3% for E_2-Q+ at concentration of 15 ng/ml and higher).

Extensive and detailed evaluation of the tissue distribution of E_2-CDS in both intact male (Chapter 5; Rahimy et al., 1988, 1990a) and OVX female rats (Chapter 6; Rahimy et al., 1990b) support the concept of brain-enhanced delivery and sustained release of E_2 using the redox-based carrier system (Bodor et al., 1987). Furthermore, the distribution patterns of both E_2-Q+ and E_2 in male (Chapter 5; Rahimy et al., 1990a) and in female rats (Chapter 6; Rahimy et al., 1990b) confirmed the major aspect of the proposed mechanism of the E_2-CDS drug delivery. Interestingly, the extent of deposition and the

chronic retention of these metabolites by the CNS are quite comparable in male and OVX female rats. The estimated half-lives of E_2-Q+ and E_2 ($t_{1/2}$ = 8-9) in brain tissue in these studies are in agreement with other reports which utilized different analytical techniques and E_2-CDS doses (Mullersman et al., 1988). Taken together these findings indicate the following: (1) as predicted based on the physicochemical properties of the E_2-CDS (Bodor et al., 1987), the "locking" of E_2-Q+ in the CNS tissue had occurred and the unique features of the BBB are the contributing factors to the chronic retention of the charged, hydrophilic E_2-Q+; (2) while the enzymatic oxidation of E_2-CDS to E_2-Q+ and the hydrolysis of E_2-Q+ to E_2 exhibit dose-dependency, the disappearance of these metabolites from the CNS tissue appear to be independent of dose. This is supported by the fact that consistent results have been obtained in several studies using doses of E_2-CDS ranging from 0.01 mg/kg (Chapter 6 & 7; Rahimy et al., 1990a, b) to 15 mg/kg dose (Mullersman et al., 1988).

Determination of distribution of E_2-Q+ and E_2 in peripheral tissues of male (Chapter 5) and female rats (Chapter 6) revealed some similarities and differences. The anterior pituitary of both male and female rats showed slower elimination of E_2-Q+ and E_2 compared with other peripheral tissues. In fact, the elimination of these metabolites from this tissue appeared more like the CNS tissue. The concentrations of E_2-Q+ and E_2 in anterior pituitary were below that of brain levels of these compounds initially and steadily decreased throughout the observation period (Chapters 5 & 6). This relative persistency of E_2-Q+ and specially of E_2 in this particular tissue are most likely because of anatomical relationship between the hypothalamus and the anterior pituitary gland. Estradiol released from the E_2-Q+, or the E_2-Q+ itself, which is "locked" into the brain, could be delivered directly to the anterior pituitary by the capillary plexus of the hypophyseal portal system (Traystman,

1983). These capillaries in the median eminence lack features of other brain capillaries and hence are not part of the BBB (Traystman, 1983).

Likewise, plasma showed, following administration of E_2-CDS to male (Chapter 5) or OVX female rats (Chapter 6), a residual but detectable levels of E_2 for approximately two weeks after a single iv dose of E_2-CDS treatment. Since E_2 is active at low pg concentrations, these plasma levels of E_2 may contribute to some of the long-term effects of E_2-CDS, i.e. uterine tissue stimulation or pituitary responses (Chapter 7). The source of this sustained residual E_2 in plasma is likely to be the result of a continuous redistribution of E_2 liberated from E_2-Q+ in the brain down its concentration gradient into the general circulation.

In contrast to CNS tissue, the distribution of E_2-CDS metabolites in male (Chapter 5) and OVX female rats (Chapter 6) showed sex differences with respect to the elimination patterns of E_2-Q+ and E_2 from certain peripheral tissues. The more obvious one was the heart tissue. Levels of E_2-Q+ in heart tissue of the male rat, following administration of E_2-CDS, were approximately 2-fold greater than levels of this metabolite in female counterparts (Chapters 5 & 6). Furthermore, the rate of elimination of this compound from heart tissue of male rat was slower than that of heart tissue of OVX female rats. Lung tissue of male rat also showed higher levels of both E_2-Q+ and E_2 than those of female rats following administration of E_2-CDS. The observed sex differences with respect to E_2-CDS metabolism and elimination patterns of these metabolites from these tissues may be related to differences in kinetics of enzymes involved. For instance, if there are tissue specific rates of E_2 metabolism that can alter the equilibrium between E_2-Q+ $\Leftrightarrow$ E_2 $\Leftrightarrow$ E_2 metabolites, that may lead to the observed differences in the disappearance of E_2-Q+ from these tissues. Furthermore, if a certain tissue, i.e.

the female heart tissue, contains E_2 inducible enzymes, i.e. cytochrome P450 monooxigenases that metabolize E_2, this may be the reason why this tissue exhibits faster clearance of E_2-CDS metabolites.

An important issue that requires further explanation is the initial distribution phase of the E_2-CDS throughout the body before its oxidation to E_2-Q+. Since the iv route was chosen here for drug administration, this route completely eliminates the process of absorption. Thus, the extent of distribution or accessibility of E_2-CDS to its ultimate site of action is determined by its ability to cross the capillary endothelial cells, then by the rate of blood flow through organs and tissues and, finally, since the parent drug likely acts intracellularly, by its rate of diffusion across the cellular plasma membranes. Since the E_2-CDS is very lipophilic in nature, it is capable of readily traversing the capillary endothelia and the plasma membranes, thus, reaching inside the cells if it survives metabolism and elimination during the initial period of distribution. Therefore, of major concerns are the general factors which may influence the amount of E_2-CDS eventually reaching and residing in different tissues. Since we do not know the extent of E_2-CDS binding to plasma proteins and other tissue components, for the present, we assume that these important issues are not critical. Thus, this general treatment of E_2-CDS kinetic behavior permits us to draw the following conclusions regarding the initial tissue distribution of E_2-CDS. First, the quantity of E_2-CDS reaching an organ or tissue represents a small fraction of the total amount of drug administered. Second, the greater the rate of blood flow to an organ or tissue the higher the quantity of E_2-CDS reaching that organ or tissue. Third, the greater the extraction efficiency from the circulation of E_2-CDS by an organ or tissue the higher the quantity of E_2-CDS that is taken up by that organ or tissue. It should be emphasized that

these factors may be important only if the E_2-CDS does not achieve an equilibrium or steady estate within 30 min after its administration. Otherwise, since the E_2-CDS does not bind to estrogen receptors itself, and since it is very lipophilic, it would be redistributed among all tissues and thus, the contribution of blood flow would be of minimal importance. However, after the oxidation of E_2-CDS to E_2-Q+, it is then the unique property of an organ or tissue, i.e. BBB, that serves the bases for retaining the oxidized metabolite within a tissue.

Several other important issues regarding the regional distribution and/or localization of the E_2-CDS metabolites, E_2-Q+ and E_2, remain to be resolved. Based upon our observations and comparisons of the whole brain and the hypothalamus (Chapters 5 & 6) and the comparisons made by Sarkar et al. (1989) of the hypothalamus, preoptic area and the cerebral cortex, it seems that the E_2-CDS is distributed evenly throughout the brain. Furthermore, no obvious differences were observed with respect to its oxidation, hydrolysis, and clearance from these brain regions evaluated to date. This suggests that the oxidation and the hydrolysis reactions leading to E_2 formation are not unique to a particular set of neurons or even other cells in the body.

Regarding the enzymatic conversion and cellular localization of the E_2-CDS metabolites, currently there are no documented reports to demonstrate that the E_2-CDS metabolism is an intracellular phenomenon. However, it is believed that the multiple enzymatic reactions are primarily an intracellular events. Using rat tissue homogenates as the test matrix, the *in vitro* observation showed a faster oxidation rate of E_2-CDS to E_2-Q+ in tissue homogenates than in plasma (Bodor et al., 1987). That suggests the involvement of membrane-bound enzyme, most likely the ubiquitous NAD

⇔ NADH transhydrogenase, in mediating the oxidation step (Hoek & Rydstrom, 1988). This enzyme/coenzyme system is primarily located in the inner mitochondrial membrane. In fact, the E_2-CDS was designed to biomimic this coenzyme moiety for its oxidative metabolism. Furthermore, since the oxidoreductive enzymes are widespread, it is expected that all tissues are capable of converting the E_2-CDS to the corresponding quaternary pyridinium salt (E_2-Q+). Likewise, the hydrolysis of E_2-Q+ to E_2 is mediated by widespread non-specific esterases present in tissues (Bodor et al., 1987; Brewster et al., 1987). We examined the disappearance of E_2-Q+ to E_2 in tissue homogenates and blood, approximately 1.2% of an E_2-Q+ dose was converted to E_2 in brain homogenate, 3.1% in whole rat blood, and 2.3% in liver after 120 min incubation of E_2-Q+. The slow rate of hydrolysis of E_2-Q+ to E_2 in an *in vitro* condition is consistent with the *in vivo* sustained release of E_2 in brain tissue over 28 days following administration of E_2-CDS in OVX female rats (Chapter 6; Rahimy et al., 1990b). Certainly, more detailed studies regarding the cellular localization of these enzymatic events and their products will lead to further insight into the understanding of mechanism of action of this delivery system.

The pharmacodynamic data demonstrates that the E_2-CDS causes dose-dependent and chronic suppression of LH and FSH secretion in OVX rats, despite the rapid peripheral clearance of the E_2-CDS metabolites (Chapter 7; Rahimy et al., 1990c). The time-course and the magnitude of gonadotropin suppression observed are quite comparable to the previously reported chronic effects of the E_2-CDS following a single iv administration to several animal models (Anderson et al., 1987a,b, 1988a,b, 1989; Estes et al., 1987a b, 1988; Sarkar et al., 1989; Simpkins et al., 1986, 1988, 1989a,b). These sustained and prolonged pharmacological effects further support the idea that the E_2-Q+ is

"locked" behind the BBB and there it serves as a brain depot for E_2 (Bodor et al., 1987).

The present work also evaluated the potential for clinical application of E_2-CDS in several estrogenically responsive animal models including androgen-dependent prostatic tissue growth/hyperplasia (Chapter 8) and morphine-dependent, naloxone-withdrawal rat model for menopausal hot flushes (Chapter 9). The encouraging results obtained from these experimental studies indicate that the E_2-CDS may have potential application for the effective treatments of androgen-dependent prostatic hyperplasia as well as vasomotor hot flushes of postmenopausal women. Although we used normal male rats (Chapter 8) as the model for prostatic adenocarcinoma to examine the effectiveness of E_2-CDS in reducing testosterone levels with subsequent regression of the prostatic tissue, there are no conclusive data as to whether this model reflects a complete picture analogous to that of the aging human prostate. However, since the primary objective of endocrine therapy in prostate malignancy is the induction of an effective androgen deprivation, thus abolishing the growth promoting effects of androgens on the diseased prostate, the E_2-CDS is quite capable of inducing an effective chemical castration-like effect (Chapter 8). Indeed, the E_2-CDS at a single dose of 0.5 mg/kg was as effective as CAST not only in suppressing T levels but also in regression of the androgen-sensitive prostatic tissue. Further experimental (animal models for prostatic adenocarcinoma) as well as clinical investigation pertaining to evaluation of therapeutic efficacy of this novel E_2-CDS in this regard as well as the menopausal hot flushes are warranted.

Finally, some of the potential complications or disadvantages of this drug delivery system need to be mentioned. Although brain-enhanced delivery with sustained release of E_2 in that tissue via a redox-based system

has potential application for various clinical conditions, it may have some drawbacks due to its mechanism of drug delivery that is, locking the drug into the CNS tissue for prolonged period of time. Potential problems may include the following: 1) The most frequent, unpleasant side effects of estrogen hormones, particularly E_2, are nausea and vomiting. These adverse effects are due most likely to their central effects. Having "locked" irreversibly a depot E_2 inside the brain, in the form of E_2-Q+, with sustained release of the hormone over prolonged period of time may cause complications in this regard. 2) In clinical practice, it is the standard procedure to monitor drug concentration in patients by taking blood samples and analyzing plasma or serum to obtain necessary informations about the drug distribution, metabolism, and perhaps drug concentration at the site of action. However, with the application of E_2-CDS, plasma or serum concentration of the E_2-CDS metabolites do not reflect the brain levels of these compounds, since these metabolites are preferentially retained by the brain. Thus, this feature of the E_2-CDS may be a disadvantage with regard to drug monitoring. 3) Certain regions of the brain, i.e. arcuate nucleus of the hypothalamus, are exquisitely susceptible to prolonged exposure and/or high concentration of estrogen hormones. Since E_2-CDS metabolites persist evenly throughout the brain for prolonged period of time after drug administration, potential for neuropathological lesions by this delivery may exist. 4) Perhaps the most important issue regarding this drug delivery would be the question of estrogen receptors down regulation or desensitization with prolonged duration of exposure to the hormone. So, if estrogen receptors down regulate, like other receptor types, the prolonged residence and the sustained release of E_2 in the brain may not be useful for the duration of residence in that tissue. And finally, although we chose the iv route for drug

administration in experimental animals in our studies, this route is not a preferred avenue of drug administration in humans. However, it should be mentioned that recently other routes of E_2-CDS administration have been examined, and it appears that oral mucosal or buccal route may be as effective as iv route for E_2-CDS administration.

In conclusion, the work presented in this dissertation demonstrate that the redox-based dihydropyridine chemical delivery system is capable of preferential delivery of E_2 to the brain by "locking" it in the form of E_2-Q^+ which then allows to be released in slow and sustained manner. The results further support the idea that the E_2-CDS may be potentially useful in fertility regulation and effective treatment of androgen-dependent prostatic diseases by virtue of selective and sustained suppression of gonadotropin secretion, and in treatment of brain estradiol deficiencies, i.e. postmenopausal syndrome. In comparison to the currently used estrogenic medications, the E_2-CDS should achieve the sustained stimulation of brain E_2 receptors at lower doses and with less frequent dosing.

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BIOGRAPHICAL SKETCH

Mohamad Hossein Rahimy was born on September 5, 1954, in Esfahan, Iran. It seems pretty old to be a graduate student at that age. But certainly it is never too late to satisfy the ego, specially with regard to knowledge. After he graduated from high school in 1974, he was compelled to serve a two-year term in the late Shah's military. Shortly after the compulsory service, he made a trip to the Florida Beach, St. Petersburg, that left a lasting impression. He entered St. Petersburg College in 1979. Two years later, he transfered to the University of Florida in Gainesville. He earned his B.S. in microbiology and cell science in December 1982. After graduation, he returned to his homeland to start his career plans, not knowing that things were changed allot. In a year or so, he decided to pursue an academic career. Thus, after his return to Gainesville in 1985, he entered the graduate program of the College of Pharmacy. In June 1985, he joined the laboratory of Dr. James W. Simpkins at the Department of Pharmacodynamics, and there he remained until the present time. Should he ever graduate, he plans to continue his research career in either pharmaceutical industry or academia.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James W. Simpkins, Chairman
Professor of Pharmacodynamics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Nicholas Bodor
Graduate Research Professor of
Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William J. Millard
Associate Professor of
Pharmacodynamics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edwin Meyer
Associate Professor of
Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ralph Dawson
Associate Professor of
Pharmacodynamics

This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1990

Dean, College of Pharmacy

Dean, Graduate School

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